University of Alberta

The Role of Interferon-γ in the Spontaneous Acceptance of Liver Allografts

by

Dr. Tina Stefania Mele ©

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Dedication

This work is dedicated to my parents, Antonio and Maria Teresa Mele, my brother Achille and my sister Rachele who have loved, supported and encouraged me throughout all my endeavors.

ABSTRACT

The ultimate goal in organ transplantation is to achieve a state of immunologic tolerance between donor and recipient, eliminating the need for immunosuppressive drugs. The spontaneous acceptance of an allograft in the absence of immunosuppression is commonly observed in experimental liver transplantation. The mechanisms mediating spontaneous acceptance remain unknown and clinically applicable strategies to achieve tolerance in human transplant recipients have been unsuccessful.

We investigated the role of IFN-γ in the spontaneous acceptance of liver allografts. We hypothesized that IFN-γ mediates a protective effect in liver allografts by induction of donor MHC class I expression which results in activation-induced apoptosis of alloreactive T cells. Transplantation of normal liver allografts into wild-type and IFN-γ-deficient recipients established that the absence of IFN-γ prevented spontaneous acceptance. Aggressive rejection of liver allografts that lacked receptors for IFN-γ confirmed that the protective effect of IFN-γ occurred via a direct effect on the graft. Histologic analysis of transplanted liver allografts demonstrated that although grafts in wild-type hosts undergo severe rejection and are infiltrated by mononuclear cells to the same extent as rejected grafts in IFN-γ-deficient recipients, grafts transplanted in the presence of IFN-γ ultimately survive long-term. Therefore, spontaneous acceptance is not due to failure of the recipient to initiate an immune response, but the result of regulation and resolution of the rejection process to allow tolerance and long-term graft survival. Rejection of MHC class I-deficient grafts in wild-type hosts verified that donor MHC class I in the graft is critical for spontaneous acceptance and may account for the

local protective effect observed in the presence of IFN-γ. We observed persistent FasL expression and increased apoptosis of recipient cells infiltrating the graft in spontaneously accepted liver allografts compared to rejecting allografts in IFN-γ-deficient hosts.

In summary, these studies establish that IFN-γ and donor MHC class I induction is critical for the spontaneous acceptance of liver allografts. In addition, our data support the hypothesis that the protective effect of IFN-γ may occur by induction of donor MHC class I which results in activation-induced apoptosis of alloreactive T cells and thus, promote spontaneous acceptance of liver allografts.

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List of Abbreviations

AICD	Antigen Induced Cell Death
APC	Antigen Presenting Cell
β2m	β-2 microglobulin
CsA	Cyclosporine A
CTL	Cytotoxic T lymphocyte
DC	Dendritic Cells
DTH	Delayed-type Hypersensitivity
GKO	Interferon-γ knockout
IFN-γ	Interferon-γ
Ig	Immunoglobulin
LMP	Latent membrane protein
mAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
MLR	Mixed Leukocyte Reaction
PCR	Polymerase chain reaction
TAP	Transporter associated with antigen processing
TAP KO	Transporter associated with antigen processing knockout
TCR	T cell receptor
Th	T helper cell
Tol-DC	Tolerogenic dendritic cells
Treg	T regulatory cell
WT	Wild-type

INTRODUCTION: A REVIEW OF TRANSPLANTATION TOLERANCE

Allograft transplantation is the transplantation of tissue or an organ from one member of a species into another, non-identical member of that same species. In 1963, Thomas Starzl performed the first successful orthotopic liver allograft in a human.[1] Since then, clinical organ transplantation has improved tremendously over the last four decades. Before 1980, the 1-year survival of patients undergoing liver transplantation was 30% or less,[2] but with improved surgical techniques and preservation solutions, along with the development of effective immunosuppressive medications, the current 1-year patient survival is greater than 85%.[3] This success is due to advancements in several areas of medicine. Understanding of the immunology involved in transplantation, the development of various surgical techniques and the discovery of immunosuppressive drugs have greatly increased the success rate of human organ transplantation. Although short-term graft survival rates are excellent, long-term graft survival is still comparatively poor due to irreversible chronic rejection and the side effects of standard immunosuppressive drugs. Liver transplantation remains the only definitive treatment for end-stage liver disease; however, the complications due to liver allograft rejection and generalized immunosuppression persist lifelong. Thousands of lives have been saved by the use of various immunosuppressive regimens but serious complications still occur as a result of the treatment. Cyclosporine A (CsA), similar to the majority of immunosuppressive drugs, causes non-specific, generalized immunosuppression which results in susceptibility to opportunistic infections and development of malignancies. In addition, CsA has several potential complications such as nephrotoxicity and hepatotoxicity. Ultimately, the goal in clinical transplantation would be to induce a state of transplantation tolerance, defined as donor-specific unresponsiveness in the absence of immunosuppressive drugs, which would avoid the problems of chronic graft rejection and generalized immunosuppression. This dream of tolerance has been driven by evidence from both experimental animal models[4] and in humans[5] that the liver itself can produce an immunologic tolerant state that may reduce or potentially eliminate the need for immunosuppressive substances.

THE HISTORY OF TRANSPLANTATION TOLERANCE

The early history of transplantation involves the intermingling of several disciplines of medicine and science including surgery, pathology, immunology, chemistry, cellular and molecular biology.

Surgical Developments in the History of Transplantation

In 1912 Alexis Carrel was awarded the Nobel Prize in Physiology or Medicine for his pioneering work on suturing of vessels and transplantation of organs. He developed a reliable method of re-anastomosing vessels that did not produce any stricture at the site of the suture. In his first publication, in 1902 in the Lyon Médical,[6] Carrel described attempts at replacing and reconstructing sections of damaged vessels by his method of suture. By persistently practicing and developing his method of suture Carrel was able to restore the circulation in complete organs which he had excised, or had replaced with other similar organs removed from another animal. In 1908, he described the successful transplantation of a kidney from one dog to another.[7] His pioneering work on the development of vascular anastomosis and organ transplantation started the pathway to successful organ transplantation. However, Carrel concluded that there was a "biological force" that prevented transplantation between individuals, and he believed that it would never be possible to succeed in having an organ from one individual function in another. Alexis Carrel received support for his belief from among others, the 1960 Nobel Laureate, Peter Medawar, who discovered the role of the immune defense system in rejection of a graft and also showed that the biological force defined by Carrel was of an immunological nature. As late as the end of the 1940s, Sir Peter Medawar, claimed that this biological force "forever will inhibit transplantation from one individual to another".

In 1990 Joseph E Murray and E Donnall Thomas received the Nobel Prize for their discoveries concerning organ and cell transplantation in the treatment of human disease. Joseph Murray developed a surgical technique for kidney transplantation in dogs and showed that a kidney transplanted from one dog to the other could be induced to function. He then later used the technique in the first successful kidney transplant between identical twins in December 1954[8] followed later by several other transplants between identical twins.[9,10] Thereafter, he proved that organ transplantation could also successfully be performed between individuals that are not genetically identical with the prerequisite of optimal immunosuppression. The discovery that ionizing irradiation and cytotoxic drugs inhibit cell proliferation made it possible to suppress the activity of the immune cells during transplantation. Murray demonstrated that total body irradiation diminished the risk of rejection of the transplanted organ. [11,12,13] About the same time George Hitchings and Gertrud Elion discovered the first cytotoxic drugs for which they were awarded the Nobel Prize in 1988. Using these early cytotoxic drugs, Murray was able to obtain improved allograft survival.[14,15] The prevention of rejection thereafter successively improved with the discovery of improved immunosuppressive drugs.

Skin Grafting in Twins

The monozygotic, "identical", twin experience starts with the treatment of burns; the dizygotic, "non-identical", twin story begins with freemartin cattle. In 1932, Dr. E. Padgett reported the use of skin allografts from family and unrelated donors to cover severely burned patients who had insufficient unburned donor sites for the harvesting of autografts. Although none of these skin allografts survived permanently, many would remain long enough to control infection and fluid loss and thus gain time for the donor sites to re-epithelialize. He observed that it was difficult to determine accurately the duration of survival of any one allograft; some seemed to melt away slowly and be replaced by adjacent skin, others seemed to be rejected rapidly. Skin grafts from family members seemed to survive longer than those from unrelated donors.[16] However, in 1937 Dr. J.B. Brown demonstrated permanent survival of skin grafts exchanged between monozygotic twins.[17] This single observation was the only ray of light in the problem of tissue and organ replacement until Gibson and Medawar demonstrated that a second allograft from the same donor was rejected more rapidly than the first.[18] This clear description of the "second set" phenomenon established that the rejection process was an allergic or immunological process which could potentially be manipulated. Grafting of normal tissue was systematically studied by Medawar who was able to show that graft rejection is a “cell-mediated” immunological phenomenon of the same nature as the tuberculin reaction and that the cellular immunological pattern is an expression of the individual genetic constitution.[19]

The concept of immunologic tolerance originated from Owen's study of freemartin cattle. Freemartins are dizygotic twin cattle in which the male is normal and the female sterile. In 1945, RD Owen[20] published an article which described the tolerogenic consequences of placental intermingling of blood between freemartin cattle. Owen defined tolerance as the absence of an immune response against a normally foreign cell to explain the inability to detect cytotoxic responses of one twin's cells against the other twin's red blood cells. Later, in 1951, Anderson and colleagues[21] reported on 2, 949 successful skin allografts between the freemartin sterile female and the normal male twin confirming the state of tolerance between the two animals. This state of tolerance observed in freemartin cattle was identified in humans when Sir Michael Woodruff, a pioneer transplant surgeon in Edinburgh and his colleague Lennox[22] found a pair of twins, one male and the other female, who shared elements of different red cell types. Postulating a shared placental circulation between the two siblings, he successfully cross skin-grafted them and thus, confirmed that the tolerant state observed in freemartin cattle was possible in humans.

Despite earlier statements regarding a biological force that "forever will inhibit transplantation from one individual to another", in 1953 Medawar along with Billingham and Brent demonstrated that acquired immunologic tolerance could be produced by neonatal injection of donor cells into a future allograft recipient.[23] Foreign tissue was inoculated into mouse embryos in the womb. The mice were later delivered at term and allowed to mature normally. These mice then accepted not only self but also foreign tissue of the same immunological pattern as that introduced during fetal life. However, they reacted as vigorously against other foreign tissues as nontreated. Thus, donor-specific immunologic tolerance, in the absence of immunosuppression, to foreign antigens would result if mice were exposed to foreign hematopoietic cells during the fetal or neonatal period. These experiments were the experimental counterpart to Owen's naturally occurring model in freemartin cattle. In 1960 Sir Frank MacFarlane Burnet and Sir Peter Brian Medawar received the Nobel Prize in Physiology or Medicine for their discovery of acquired immunological tolerance. These initial observations have now been repeatedly confirmed and expanded in various studies. Although not directly clinically applicable to adult transplantation, experimentally produced tolerance has developed into a biological research tool of great usefulness.

Advances in Cellular and Molecular Biology

In 1980 Baruj Benacerraf, Jean Dausset and George D Snell were awarded the Nobel Prize for their discoveries concerning MHC antigens on the cell surface that regulate immunologic reactions. The surface of all body cells is unique in every individual. This unique character is determined by genes that regulate the formation of specific protein-carbohydrate complexes, the major histocompatibility (MHC) antigens, found on the cell membrane. George Snell laid the foundation for our knowledge of the laws that govern the body's ability to distinguish "self" from "non-self". Snell developed mouse strains that through repeated sibling mating were made genetically identical. Snell then transplanted tumor cells from cancerous to healthy mice and found that transplanted tumors grew progressively in all mice of the same strain, but were rejected in foreign strains. Crossing experiments showed that transplanted tumor cells could only grow if the donor and the recipient shared certain dominant genes. Snell realized that the reaction was not limited to cancer cells, but that the transplantability of normal tissues was regulated by the same genes. Snell called them "histocompatibility genes" or H-genes. Snell showed that these genes determined the presence of cell surface antigens which he later named major histocompatibility (MHC) antigens. With Snell's fundamental discoveries came the birth of transplantation immunology.

Although Snell introduced the concept of MHC antigens, it was Jean Dausset who demonstrated the existence of MHC antigens in man and elucidated the genetic factors regulating their formation. Between 1930 and 1950 when knowledge about transplantation immunology was increasing in the mouse, nothing was known about a corresponding system in man. At the time when Dausset started his activities in this field, it was already clear that humans reject foreign grafts by the same type of immune mechanism as mice. Experimental tissue transplants comparable to those practiced on laboratory animals were not possible. Originally, Dausset studied autoimmune diseases, and one of his methods was through immunological investigations of patients who had undergone repeated blood transfusions. He found that patients who received many blood transfusions produced antibodies that killed white blood cells. At first, he thought that this was an autoimmune reaction, i.e. that the patients reacted against their own white cells. However, this did not fit with the fact that the white cells of the blood donors were killed, but the cells of the recipient remained unharmed. Dausset realized that he had encountered a previously unknown type of genetic variation between people. He went on to study the antibodies of women who had given birth to several children. On the basis of family analyses, he demonstrated that the variation was determined by a single genetic system, localized to a single chromosome. The variation was designated human leukocyte antigens (HLA), and the genes that determined their formation, HLA genes. The HLA genes were found to be analogous with H-2 genes in the mouse. Thereby Dausset had identified the human equivalent to the H-genes in mice. Dausset showed that within the HLA system in man there were two dominating regions, and Snell was subsequently able to show that this was the case as well with the H system in mice. At this point, the paths of Snell and Dausset converged. Research on mice and humans became mutually complementary. Dausset's discovery had many practical applications. With the aid of his system it is possible to tissue type both donor and recipient, thereby considerably increasing the likelihood of a successful transplant.

IMMUNOLOGIC BASIS OF ACUTE ALLOGRAFT REJECTION

Spontaneous acceptance of liver allografts occurs despite an initial acute rejection process which eventually resolves. To identify the mechanisms mediating spontaneous acceptance, it is necessary to understand how allograft rejection normally occurs and how this process is modified to allow graft acceptance without the need for immunosuppression.

T Cells Mediate Acute Allograft Rejection

T lymphocytes play a central role in the generation of alloimmune responses and orchestrate both allograft rejection and tolerance. During the early 1940s Medawar demonstrated the immunologic basis of allograft rejection while working with burn patients during World War II. Medawar noted that skin grafts from one site to another on the same patient were accepted, while donor grafts from another patient were rejected. By carefully studying the histology of skin grafts, Medawar observed that allografts were infiltrated by lymphocytes and monocytes of donor origin and thus concluded that graft rejection is a "cell-mediated" immunological phenomenon of the same nature as the tuberculin reaction.[24,25,26] During rejection a vascularized graft becomes infiltrated with lymphocytes, monocytes and other inflammatory cells. By 6 to 9 days following transplantation, there is decreased vascularization of the transplanted tissue, visible necrosis by 10 days and complete rejection by 14 days. He also noted that prior sensitization of the recipient with donor cells or a previous skin graft led to accelerated rejection of a second allograft from the same donor.

In 1954 Mitchison demonstrated that lymph node and spleen cells harvested from tumor allograft recipients could adoptively transfer immunologic resistance to tumor allografts, whereas transfer of serum did not have the same effect.[27] Using a similar approach, Billingham et al[28,29] showed that immunity to a skin allograft could also be adoptively transferred by lymph node and spleen cells. Later studies began to implicate T cells as the primary cellular mediators of allograft rejection. Hall et al demonstrated that when cell populations were transferred to sublethally irradiated rats carrying heart allografts, long-lived, re-circulating, immunoglobulin-negative cells were responsible for initiating rejection.[30] Furthermore, nude mice which lack a thymus and consequently, lack functional T cells, are unable to reject an allograft.[31,32] Analysis of the T cell populations involved in allograft rejection has implicated both CD4+ and CD8+ cells. In a study using depleting monoclonal antibodies, removal of CD4+ T cells prolonged graft survival from 15 days to 30 days. Although removal of CD8+ T cells alone had no effect on graft survival, removal of both CD4+ and CD8+ T cells had the most pronounced effect on graft survival (up to 60 days).[33] Both delayed-type hypersensitivity and cell-mediated cytotoxicity reactions have been implicated. The process of graft rejection can be divided into two stages: (1) a *sensitization phase* in which recipient CD4+ and CD8+ T cells recognize alloantigens expressed on cells of the foreign graft and proliferate in response and (2) an *effector phase* in which immune destruction of the graft takes place.

The Role of MHC in Graft Rejection

Self-MHC Restriction

With the ground work laid by Snell and Dausset, Zinkernagel and Doherty were able to demonstrate the requirement for T cells to recognize simultaneously both 'foreign' molecules and self molecules (major histocompatibility antigens).[34,35] Studies by Zinkernagel and Doherty demonstrated that T cell effector function is generally self-MHC restricted such that T cells will only be activated upon recognition of a foreign antigen presented within the groove of a "self-selected" MHC molecule.[36] The majority of "self-selection" occurs during fetal maturation where cells bearing MHC molecules are either "positively" selected if they fail to elicit a response when presenting "self" antigens to cells within the thymus or "negatively" selected if they generate too much of a response. This process is a type of "check system" that prevents the immune from reacting and generating a response to "self" antigens. This discovery provided insight into the antigen recognition mechanisms of T cells within the immune system. T cells must distinguish "self" antigens from "foreign" antigens to prevent autoimmunity or the destruction of "self". Therefore, as a protective mechanism, activation of T cells can only occur upon recognition of foreign peptide presented within the context of the peptide-binding groove of "self"-MHC molecules. For their significant contribution, Zinkernagel and Doherty were awarded the Nobel Prize in Physiology or Medicine in 1996.

Alloantigen Recognition

The initiating event in any alloantigen-specific immune response, including both rejection and tolerance, is alloantigen presentation. Graft rejection is caused principally by a T cell-mediated immune response to alloantigens, primarily MHC molecules, expressed on the surface of cells within the graft. Recipient T cell activation can be initiated by recognition of donor allogeneic peptides presented on self-MHC molecules by recipient APCs or by intact allogeneic donor MHC molecules expressed on donor APCs, the indirect and direct pathways, respectively. The combination of these two forms of allorecognition, which take place primarily in draining lymph nodes, accounts for the high proportion of effector T cell precursors involved, the tremendous strength of the alloimmune response, and the lack of need for priming. Alloantigen-activated T cells subsequently expand, acquire effector properties, and migrate into the graft resulting in rapid graft damage and destruction.

The initiation of allograft rejection is thought to occur via the direct pathway which involves recognition of intact donor MHC class II moleculues on donor APCs by recipient CD4+ T cells. Activation of CD4+T cells in response to alloantigen MHC occurs independent of the peptide within the MHC peptide groove. Direct allorecognition is triggered by passenger dendritic cells (DC) of donor origin which migrate out of the graft early after transplantation to local lymph nodes where they encounter the recipient immune cells.[37,38] These passenger DC express high levels of class II MHC molecules and are widespread in most mammalian tissues. Because passenger leukocytes express the allogeneic MHC antigens of the donor graft, they are recognized as foreign and therefore, stimulate immune activation of T cells in the lymph node. As many as 5% of recipient T cells will recognize and react via the direct pathway to an allogeneic MHC molecule-peptide complex. Direct alloantigen recognition is the main cause of early acute rejection episodes following transplantation. The indirect pathway involves recipient CD4+T cell recognition of donor MHC molecules which have been processed and are presented as peptides bound to MHC class II molecules displayed on the surface of recipient APCs.[39,40,41,42] Recipient APCs can also migrate into a graft, endocytose the foreign alloantigens and present them as processed peptides together with self-MHC molecules.

Effector Stage of T cell Activation

Effector T cells are generated in the regional nodes and are carried by the lymphatics back to the graft to mount an immunologic attack. Activated recipient CD4+ T cells and APCs produce cytokines that enhance lymphocyte proliferation and the maturation of CD8+ cytotoxic T cells specific for donor class I MHC molecules. A variety of effector mechanisms participate in allograft rejection. The most common are cell-mediated reactions involving delayed-type hypersensitivity and cytotoxic T lymphocyte (CTL)-mediated cytotoxicity. Less common mechanisms are antibody-plus complement lysis and destruction by antibody-dependent cell-mediated cytotoxicity (ADCC). The hallmark of graft rejection involving cell-mediated reactions is an influx of T cells and macrophages into the graft. Histologically, the infiltration in many cases resembles that seen during a delayed-type hypersensitivity (DTH) response in which cytokines produced by T_{DTH} cells promote macrophage infiltration. Recognition of foreign class I alloantigens on the graft by host CD8+ cells leads to CTL-mediated killing. In each of these effector mechanisms, cytokines secreted by Th cells play a central role. For example, IL-2, IFN-γ and TNF-β have each been shown to be important mediators of graft rejection. IL-2 promotes T cell proliferation and generally is necessary for the generation of effector CTLs. IFN-γ is central to the development of a DTH response, promoting the influx of macrophages into the graft and their subsequent activation of more destructive cells. A number of cytokines promote graft rejection by inducing expression of class I or class II MHC molecules on graft cells. During a graft rejection episode, the levels of these cytokines increase, inducing a variety of cell types within the graft to express class I or class II MHC molecules and thus, promoting the immune response against the graft.

Clinical and Histologic Manifestations of Liver Allograft Rejection

Allograft rejection is divided into hyperacute, acute and chronic rejection. Hyperacute rejection, which occurs within the first 24 hours after transplantation, is due to pre-existing antibodies to graft antigens resulting from previous blood transfusions, pregnancy, or previous grafts. It is characterized by loss of vascular integrity, leading to interstitial hemorrhage, edema and infiltration of neutrophils. Liver allografts appear to be relatively resistant to antibody-mediated, hyperacute rejection.[43] Acute rejection generally begins during the first few weeks after graft transplantation. It is usually a cell-mediated immune response characterized by graft infiltration of macrophages and lymphocytes. Chronic graft rejection can occur months to years post-transplantation. This type of rejection is thought to be mediated by both humoral- and cell-mediated responses.

Histologic examination of a liver allograft correlates the tempo of rejection to the severity of liver architecture disruption and intensity of cellular infiltration of the portal tracts and sinusoids.[44] Examination of a liver allograft 1-2 weeks post-transplantation reveals infiltration of portal tracts, central venules and sinusoids by lymphocytes, macrophages, and plasma cells. Immunohistochemistry staining demonstrates that the infiltrating cells are predominantly T cells and monocytes in the portal tracts, with vascular subendothelial and biliary epithelial aggregates. Eosinophils and polymorphonuclear leukocytes are present to a lesser degree but are more prevalent in hepatic rejection than in cellular infiltrates seen in other organs. The morphologic changes include edema of the portal tracts, bile duct proliferation and hepatocyte necrosis. Bile duct proliferation is a common finding in liver isografts and allografts and may be reflective of biliary obstruction and not immunologic damage.[45,46]

The location of the cellular infiltrate corresponds to the expression of MHC class I and class II antigens. MHC class I antigens are present on biliary epithelium, sinusoidal lining cells, vascular endothelium and weakly expressed on hepatocytes. MHC class II antigens are also present on the biliary epithelium and sinusoidal lining cells, but absent on hepatocytes and the vascular endothelium. Interstitial DC observed around the bile ducts and portal triad also express MHC class II antigens. Kupffer cells express both class I and class II antigens.[47,48,49,50,51,52,53] The fact that hepatocytes express low levels of MHC class I and no MHC class II (even in the face of acute rejection) has been suggested as a explanation for the relative the resistance of liver allografts to the immune response because there would be no induction of MHC expression on hepatocytes with INFγ. In rat and mouse strain combinations with spontaneous allograft acceptance, there is histologic evidence that similar changes occur during the first two weeks post-transplantation. However, histologic examination at 4 weeks post-transplantation reveals that the sinusoidal mononuclear cells have disappeared and by 3-4 months post-transplantation the initial inflammatory changes have completely resolved.[54] Therefore, spontaneous acceptance is not due to failure of the recipient to initiate an immune response in the early post-transplant period, but the result of regulation and resolution of the rejection process to allow long-term survival and tolerance of the graft.

THE ROLE OF INTERFERONγ IN TRANSPLANTATION

MHC Class I and Class II Expression

MHC class I expression is essential in the immune response because they present antigen-derived peptides to cytotoxic T lymphocytes. MHC class I molecules are ubiquitously expressed and their basal level of expression can be induced by a number of cytokines and viruses.[55] Expression of MHC class I molecules is tightly regulated at the transcriptional level during development and also in fully differentiated cells by transcriptional factors binding to *cis*-acting regulatory elements within the MHC class I promoter. The main control elements include enhancer A, the interferon-stimulated response element (ISRE), and site α.[56,57] Enhancer A is bound by transcription factors of the NF-κB/rel family and is thought to be essential for constitutive and cytokine–induced expression.[58] The ISRE is the target DNA-binding site for factors of the interferon regulatory factor (IRF) family and it mediates the induction of MHC class I expression by type I and type II interferons.[59] Interferon-γ (IFN-γ) is a potent inducer of MHC class I membrane expression. The IFN-γ induction pathway that leads to MHC class I activation is initiated by the binding of IFN-γ to its receptor, which leads to the activation of the tyrosine kinases JAK1 and JAK2. These tyrosine kinases phosphorylate the signal transducer and activator of transcription-1α (STAT1α; also termed p91, STAT91 or GAF). STAT1α binds the γ-activated site of genes, such as interferons regulatory factor-1 (IRF-1) as a homodimer inducing transcription of these IFNγ-inducible genes. [60,61,62,63]

IRF-1 is the principal transcription factor which binds to the ISRE in the MHC class I promoter.[64,65,66] Site α is highly conserved among various MHC class I loci, with the exception of HLA-G.[67] It has been postulated that proteins of the Fos/Jun or ATF/CREB family of transcription factors bind to this site. [68,69] Gobin et al demonstrated that members of the ATF/CREB family of transcription factors bind to site α in a constitutive manner, whereas, proteins of the Fos/Jun family of transcription factors could not be detected. Using transient transfection experiments, Gobin et al, and later, other investigators, demonstrated that site α is essential for constitutive MHC class I expression and IFNγ-induced activation of MHC class I through the ISRE.[70] In addition to the MHC class I heavy chain, IFNγ also enhances expression of β_2m, TAP1, TAP2, LMP2 and LMP7[71] all of which play an important role in MHC class I-mediated antigen presentation.[72]

Transcriptional activation of MHC class II genes requires class II transactivator (CIITA).[73] CIITA is involved in IFNγ-induced MHC class II transactivation. MHC class II gene promoters contain a set of conserved regulatory elements, known as the S (W or Z), X1, X2 and Y boxes. These regulatory elements are bound by a number of DNA-binding proteins including the RFX protein complex, which binds the X1 box; X2BP and other Fos/Jun- and ATF/CREB-related proteins, which bind the X2 box; and NF-Y, which binds the Y box.[74] CIITA is thought to act as a coactivator and since no DNA-binding motiff has been found, CIITA is believed to exert its activity through these DNA-binding proteins.[75] In particular, the S and X2 boxes and their DNA-binding proteins appear to be crucial for mediating transactivation by CIITA.[76] CIITA contains several domains involved in MHC class II activation: an N-terminal acidic domain with transactivation properties; a proline-, serine-, and threonine-rich domain; and a C-terminal domain. CIITA is constitutively expressed in MHC class II-positive cells such as B cells. In other cell types, the expression of CIITA can be induced by IFNγ and is under the control of the IFNγ-mediated signal transduction pathway.[77] Therefore, MHC class II genes are inducible by IFNγ, despite a lack of ISRE and γ-activated site elements in their promoter region.

Protection versus Promotion of Rejection of Allografts

IFN-γ has traditionally thought to be a promoter of immune responses. IFN-γ affects many aspects of the host immune response, including cytokine production, Ab production, and CTL generation. Indeed, allograft rejection is associated with intense production of IFN-γ, which acts in the graft and on host cells. As described above, one manifestation of IFN-γ action on the graft is the induction of MHC class I and II expression, the major alloantigens, in the parenchyma of the rejecting organ[78,79] and in the host tissues.[80,81] The requirement for IFN-γ during the rejection process is supported by several studies in experimental animal models. IFN-γ is required for rejection of established islet transplants by CD8 T cells in a TCR-transgenic model,[82] for rejection of class II-disparate skin grafts,[83] and aggravates chronic vascular injury in heart transplants.[84,85]

Yet despite the association of IFN-γ with inflammation and MHC regulation, recent evidence has emerged to suggest a protective role for IFN-γ during allograft transplantation. Mice with disrupted IFN-γ genes reject transplants briskly.[86,87,88] IFN-γ deficiency does not prevent myocardial rejection in transplanted mouse hearts,[89] and mice lacking IFN-γ receptors reject islet transplants.[90] Examination of kidney allografts from donors that lack IFN-γ receptors demonstrated that these transplants undergo massive necrosis beginning at days 5–7 which does not occur in allogeneic transplants with intact receptors for IFN-γ.[91] The massive necrosis was likely due to ischemia secondary to microvascular injury and congestion. Similarly, vascularized heart or kidney allografts rejecting in IFN-γ recipients demonstrate rapid development of necrosis, with congestion and small thrombi in veins, but with patent large vessels. It appears that the predominant early role of IFN-γ in rejection of vascularized organs such as kidney and heart is protection against early failure of the microcirculation and necrosis, probably by a direct action on the graft.[92] The surprising efficiency of graft rejection in mice lacking IFN-γ has been attributed to the ability of IFN-γ to inhibit lymphocyte proliferation and CTL generation.[93] Heart allografts are rejected by IFN-γ-deficient hosts using either a CD4-dependent pathway or a novel CD8-dependent, CD4-independent pathway which is not suppressed by anti-CD40 ligand.[94] Moreover, IFN-γ plays a role in protecting the graft against early failure in concordant rat to mouse xenotransplants.[95] The ability of IFN-γ produced by recipient cells to minimize necrosis in the grafted parenchymal tissue despite florid rejection may explain why IFN-γ is needed in some tolerance protocols.[96,97,98] Thus, in the process of rejection of allografts or concordant xenografts, IFN-γ displays diverse effects, many attributable to the immunoregulatory or effector activities of IFN-γ.

EXPERIMENTAL ANIMAL MODELS OF LIVER TRANSPLANTATION

Nonvascularized skin grafts were the only model available to study transplant immunology until 1961 when Lee and Fisher developed a portocaval shunt.[99] This was followed by the development of a renal transplant model in rats which facilitated further study of allografts in experimental animal models.[100]

Large animal models

Experimental liver transplantation techniques were first described in the dog[101,102,103,104] and soon after, in the pig.[105] In 1966, Cordier et al reported spontaneous prolonged survival of orthotopic liver allografts without immunosuppression in the pig.[106] Calne further demonstrated that whereas skin and kidney allografts in pigs were uniformly rejected, some liver allografts were not. In general, he noted that liver grafts in all species were rejected less aggressively than other organs such as the heart, kidney, and pancreas.[107] Histologic examination of grafts with prolonged survival demonstrated evidence of rejection, repair and regeneration. The intensity of the cellular infiltrate correlated to the tempo of rejection.[108]

Small animal models

In 1973, Lee et al[109] described the first rat model of OLT with hepatic arterialization. To simplify the procedure, a second, non-arterialized model was developed.[110] As a result, two models of rat OLT are currently used for organ transplantation research; one with dual blood flow from the hepatic artery and portal vein (arterialized model), and the second model supported solely by portal venous blood flow without hepatic arterialization (non-arterialized model). Later, Kamada et al[111] introduced the cuff technique for reanastomosis of the portal vein, infrahepatic vena cava and reconstruction of the common bile duct over a polyethylene stent. This method allowed shorter clamping time of the portal vein which resulted in improved long-term survival.

Zimmerman[112,113] and others,[114] observed that in certain rat strain combinations, liver allografts had prolonged survival despite fully allogeneic MHC barrier, whereas similar kidney and heart allografts underwent acute rejection without exception in less than 15 days. Although spontaneous acceptance was dependent on the combination of strains selected, the hyporesponsiveness observed was donor-specific with appropriate third-party immune responses intact. In addition, recipients who accepted a liver allograft accepted subsequent donor-specific heart, skin or kidney transplants that would not have been otherwise accepted.[115,116] This observation of liver mediated, donor-specific tolerance has been well described in the pig and rat model.[117,118,119] PVG rats grafted orthotopically with a DA liver have been shown to accept subsequent DA skin grafts[120,121] and simultaneous or subsequent grafts of DA heart[122] or kidney.[123] Peripheral lymphocytes of liver-grafted rats transferred to irradiated syngeneic recipients bearing donor-type or third-party skin grafts displayed normal reactivity against the third-party skin graft and were completely without effect on the donor-specific skin graft. In all cases, tolerance was demonstrated to be specific for antigens of the liver donor by rejection of appropriate third party grafts.

In 1991, Qian and colleagues published the first series of successful liver transplants in a mouse model.[124] A mouse model of orthotopic liver transplantation offers significant advantages for immunologic research due to the well-defined mouse genome with numerous commercially available, genetically-defined inbred, transgenic, and knockout mouse strains. In addition, the availability of molecular probes, monoclonal antibodies, and reagents specific for the mouse species combined with decreased costs associated with purchasing and housing facilitate research using mouse models.

SPONTANEOUS ACCEPTANCE OF LIVER ALLOGRAFTS

Spontaneous acceptance is the acceptance of an allograft in the absence of immunosuppression. Although spontaneous acceptance has been documented in many experimental and human transplants, mouse liver allografts have long been recognized to have a relatively high spontaneous acceptance rate.[125,126] A liver allograft is more acceptable to its host than are grafts of other organs such that a liver grafted between widely disparate species can be tolerated for days, whereas xenografts of heart or kidney will only accept perfusion with recipient blood for a fraction of an hour.[127] In pigs, rats and mice, although destructive rejection can occur, prolonged liver allograft survival, in the absence of immunosuppression, is commonly observed. However, in the dog, baboon, rhesus monkey and man, immunosuppressive treatment is necessary and even with full doses of conventional agents, uncontrollable rejection can lead to death from liver failure. Although, there is evidence that even in these species the liver is less aggressively rejected than are other organs. In addition, spontaneous acceptance of a liver allograft is associated with a state of donor-specific tolerance where heart, kidney or skin grafts from the same donor origin can be transplanted and accepted without the need for immunosuppression. This state of donor-specific tolerance is observed more frequently in human liver transplant recipients that with any other organ.[128,129] Among Starzl's longest surviving liver recipients, now many years post-transplantation, 12 (28%) patients have been without immunosuppressive medications for as long as 16 years.[130]

Since the early descriptions of immunologic tolerance, a large number of strategies have shown to be capable of achieving allograft tolerance in experimental models. However, most attempts at bringing these strategies into clinical use have been unsuccessful, and although 'operationally' tolerant human recipients who have discontinued their immunosuppression have been reported, particularly in liver transplantation, we still lack an acceptable therapeutic protocol to induce clinical allograft tolerance. Although there has been considerable research in the area of spontaneous acceptance, the critical events involved still remain to be elucidated. Identifying the mechanisms mediating spontaneous acceptance of liver allografts may facilitate development of donor-specific immunosuppressive strategies which could potentially avoid the complications associated with generalized immunosuppression and chronic graft rejection and ultimately, improve long-term survival of human recipients of liver allografts.

POTENTIAL MECHANISMS OF SPONTANEOUS ACCEPTANCE

Spontaneous acceptance of liver allografts in certain rat strain and many mouse strain combinations is not well understood. Histologic examination of allografts supports the concept that spontaneous acceptance is the result of initial rejection with resolution of the process. This is consistent with an active process that has resolved or is in a constant state of homeostasis/suppression. Furthermore, many experiments, specifically skin graft data, demonstrate that it is a donor-specific tolerant state. Several mechanisms have been postulated to explain the events which lead to donor-specific tolerance.

Hepatocyte release of Soluble MHC Molecules

Calne and others have postulated that an induced state of donor-specific hyporesponsiveness could be mediated by soluble MHC class I antigens produced by the grafted liver.[131,132] The liver may provide such large quantities of soluble antigen that the antibody-mediated humoral response is "sopped up" or neutralized by all this soluble antigen. In support of this hypothesis is the observation that in rats and in humans, circulating MHC class I molecules of donor origin have been detected in the serum of liver allograft recipients.[133,134] In addition, several investigators have demonstrated that crude extracts of liver, administered before grafting and combined with immunsuppressive regimens not highly effective on their own, induced specific unresponsiveness that permitted long-term survival of donor-strain skin allografts.[135,136]

Clonal Deletion of Alloreactive T cells

Some investigators have postulated that acceptance of liver grafts is due to deletion of alloreactive cells. Despite in vivo hyporesponsiveness to liver allografts, splenocytes or liver lymphocytes from mice with an accepted liver allograft display donor-specific reactivity in vitro during mixed leukocyte reactions and cytotoxicity assays. Thus, clonal deletion is not a likely mechanism responsible for this "split tolerance" phenomenon[137,138]

Chimerism

Starzl et al have proposed that allograft tolerance observed in solid organ transplantation is mediated by donor nonparenchymal cells which migrate out of the graft leading to subsequent donor/recipient chimerism.[139] Mixed chimerism was first observed by Kashiwagi et al[140] in 1969 with karyotyping studies in female recipients of male livers. Post-transplantation, the hepatocytes and the endothelium of the major blood vessels of the liver grafts retained their donor-derived cells, but the macrophages, including the Kupffer cells, were replaced by recipient cells.[141] Starzl and others have demonstrated the presence of donor-derived cells in kidney and liver recipients with long-term allograft acceptance.[142,143] Several studies have demonstrated persistence of donor-derived cells in patients with long-term surviving grafts using polymerase-chain reaction (PCR) analysis. Furthermore, Alard et al demonstrated, using PCR analysis, that levels of chimerism appeared higher in tolerant animals than in animals who rejected their grafts.[144] However, it remains unclear whether microchimerism is the cause or a consequence of the induction of tolerance.

Anergy

Interference with costimulation during T cell receptor engagement of the MHC-peptide complex results in antigen-specific hyporesponsiveness or anergy. Anergy refers to a functional state of hyporesponsiveness in which the cell is unable to produce IL-2 and other cytokines after antigen recognition.[145,146,147] This anergic state was first described by investigators using mouse T_h[148] and human T cell clones,[149] and later with freshly isolated T cell populations.[150] These systems provide a tissue culture model for the state of *in vivo* tolerance known as T cell clonal anergy, in which self-reactive lymphocytes that are not deleted during development cannot be activated to divide upon stimulation with antigen and presenting cells.[151] The classical model for anergy induction made use of mouse T cell clones triggered by antigenic peptides either on chemically fixed APC or in planar lipid membranes containing MHC II molecules.[152] When chemically fixed spleen cells were used as APCs, anti-CD28 monoclonal antibody could prevent anergy induction. Cytotoxic T lymphocyte Ag-4 (CTLA-4) was discovered in a cDNA library of T cell-specific, activation-induced genes.[153] CTLA4, a glycoprotein homologous to CD28, is expressed on activated CD4+ and CD8+ T cells[154,155] and binds to the same ligands, B7-1 and B7-2, as CD28 but with a 20- to 50-fold higher affinity.[156,157] A soluble fusion protein of human CTLA-4 and the heavy chain Fc region of IgG1 binds to both B7.1 (CD80) and B7.2 (CD86) with high affinity (20-fold greater affinity than CD28). Administration of CTLA4Ig effectively blocks the interaction of CD28 with either B7.1 or B7.2, and thus, inhibits this critical costimulatory pathway, prevents T cell activation, and induces T cell unresponsiveness *in vitro*.[158,159] *In vivo* blockade of B7, using B7 antagonists such as anti-B7 monoclonal antibody or CTLA4Ig, has been shown to prolong the survival of allografts and xenografts in vivo.[160,161]

Tolerogenic Dendritic Cells and the Passenger Leukocyte Theory

Leukocytes resident in the liver may play a role in immune responses and as such, the "passenger leukocyte" theory was brought forth as a possible explanation for spontaneous acceptance of liver allografts. Initially proposed by Snell in 1957,[162] the "passenger leukocyte" theory states that donor leukocytes within the grafted tissue are a major source of tissue immunogenicity. This theory was consistent with the observation that immunization of a recipient animal with donor spleen or lymph node cells would sensitize the animal to a tumor allograft, whereas antigen extracts of the tumor were only weakly immunogenic and, under certain conditions, would promote the growth of the tumor graft.[163] Evidence to suggest that passenger leukocytes could mediate graft acceptance comes from Steinmuller who initially rendered strain A mice neonatally tolerant to strain B mouse antigens. He then transplanted skin isografts from these tolerant strain A mice onto naive strain A mice. He was able to demonstrate that these naive strain A mice, once skin-grafted, become tolerant to strain B antigens and were able to accept strain B allografts.[164] These neonatally tolerant mice were hematopoietic chimeras and thus, it was postulated that the leukocytes within the skin isografts, tolerant to both A and B antigens, were able to passively transfer strain B immunity to the naive strain A mice.

Passenger donor leukocytes within liver allografts are mostly bone marrow derived cells.[165,166,167] T cell differentiation is regulated by the local microenvironment. Hence, the property of antigens encountered by the T cell, and the expression of costimulatory molecules and cytokines by APCs drive T cell differentiation. In vitro, IL-12 drives Th1,[168,169] whereas IL-4 promotes Th2 differentiation.[170,171] Similarly, the generation of T regulatory 1 (Treg 1) cells is driven by IL-10.[172] The stimulus controlling T cell differentiation during an in vivo immune response is less clear. DC are uniquely suited for activation of naive T cells.[173] Recent data suggest that different DC subsets provide T cells with selective signals that guide either Th1 or Th2 differentiation. In mice, DC have been classified into myeloid and lymphoid subsets according to their phenotype and their development from distinct precursors.[174,175,176,177,178] These subsets of DC share a number of distinct properties, including dendritic morphology, the ability to migrate, and expression of a range of molecules required for activation of naive T cells. However, they differ in their regulation of the immune response. Myeloid DC usually initiate immune responses, and typically induce Th1 differentiation. In contrast, the so-called lymphoid DC propagated in response to IL-3, while capable of activating lymphocytes, may also limit T cell proliferation by inducing Fas-mediated apoptosis and inhibiting cytokine production.[179,180,181,182] Analogous to mice, humans may also contain two DC types developed from distinct precursors. DC1, propagated in response to GM-CSF from peripheral blood monocytes, produce high levels of IL-12 and induce Th1 differentiation. On the other hand, DC2 propagated from blood or tonsil plasmacytoid T cells in response to IL-3, drive Th2 differentiation.[183,184] Furthermore, repetitive stimulation with allogeneic immature DC induces IL-10-producing, nonproliferating T cells with regulatory properties.[185]

Dendritic cells have been shown to play a role in spontaneous acceptance of liver allografts. Lu et al described a novel cell population propagated from mouse liver nonparenchymal cells in response to IL-3 and anti-CD40 mAb that exhibit a distinct surface immunophenotype and function in directing differentiation of naive allogeneic T cells.[186] After culture, such cells are DEC-205^{bright}B220^{+}CD11c^{-}CD19^{-}, and negative for T (CD3, CD4, CD8α), NK (NK 1.1) cell markers, and myeloid antigens (CD11b, CD13, CD14). These liver-derived DEC205^{+}B220^{+}CD19^{-} cells have a morphology and migratory capacity similar to dendritic cells and express the DC marker DEC205.[187] Interestingly, they also bear the B220 Ag, a marker of cell activation typically associated with B cells, but do not express the B cell Ag CD19. These cells also possess Ig gene rearrangements, but lack Ig molecule expression on the cell surface. They induced low thymidine uptake of allogeneic T cells in MLR due to extensive apoptosis of activated T cells. T cell proliferation was restored by the addition of the common caspase inhibitor peptide, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk). T cells stimulated by liver-derived DEC205$^+$B220$^+$D19$^-$ cells release both IL-10 and IFN-γ, small amounts of TGF-β, and no IL-2 or IL-4, a cytokine profile resembling Treg1 cells. After *in vivo* administration of liver DEC205$^+$B220$^+$CD19$^-$ cells into allogeneic recipients, they migrate to spleen and dramatically prolong a subsequent cardiac allograft survival.

DC suppression of T cell responses can occur through weak costimulation[188,189] or through activation of Treg.[190,191] Tolerogenic DC (Tol-DC) encompasses DC subsets that possess the immature phenotype of low expression of MHC class II, CD40, CD80, CD86, and IL12 and high expression of IL10.[192,193,194] Tol-DC include lymphoid, CD8α+ DC that direct Th2 differentiation,[195] immature myeloid DC that activate Treg[196] and hepatic DC that possess weak costimulatory molecules and are believed to contribute to the low immunogenicity of hepatic allografts.[197] *In vitro* studies have demonstrated that T cells that secrete TGF-β and IL10 can promote the generation of Tol-DC with immature phenotype.[198,199] Tol-DC have been used therapeutically to prevent allograft rejection.[200,201,202]

T regulatory cells

Several investigators have proposed that a specific subset of T cells act to suppress or regulate the alloimmune response which leads to donor-specific transplant tolerance. Accumulating evidence in experimental models indicate that the balance between alloaggressive and regulatory T cells is one of the key points in the decision between graft rejection and immunological tolerance. This phenomenon of T cell mediated regulation has been long known, but only recently have regulatory T cells (Tr_{eg}) been characterized as CD4+CD25+ T cells and their role in the induction and maintenance of transplantation tolerance more carefully defined.[203,204,205,206] These cells increase in number during transplant tolerance and can transfer tolerance in an antigen-specific manner to secondary recipients.[207] Although, Treg cells are generated intrathymically during development,[208] their production can also occur in the periphery due to interaction between naive T cells and subsets of dendritic cells which are collectively referred to as tolerogenic DC (Tol-DC).[196, 209, 210]

Min et al demonstrated the induction of donor-specific transplant tolerance in a fully MHC-mismatched murine model of cardiac transplantation was achieved by simultaneous induction of Treg and Tol-DC with the administration of anti-CD45RB mAb and LF 15-0195, respectively.[211] LF is a novel analog of the antirejection drug 15-deoxyspergualin (DSG) which induces donor-specific tolerance.[212] DSG, the parent compound, specifically inhibits the maturation of DC.[213] Increased number of splenic Treg and Tol-DC were observed in tolerant recipients as assessed by an increased in CD4+CD25+ T cells and immature DC. Tol-DC purified from tolerant recipients incubated with naive T cells induced the generation of CD4+CD25+ Treg. In addition, incubation of Treg isolated from tolerant recipients with DC progenitors resulted in the generation of DC with the Tol-DC phenotype. Based on this study, tolerance induction is associated with a self-maintaining regulatory loop in which Tol-DC induce the generation of Treg from naive T cells and Treg programs the generation of Tol-DC from DC progenitors. Another possibility is the induction of T reg cells[185] by a mechanism independent of DC. Several varieties of Treg cells have been described, each with unique characteristics. Thus, a number of definitions of Treg cells exist in the literature.[185,196, 214] Type 1 Treg (Treg1) cells are a subset characterized by their unique profile of cytokine production. Treg1 cells produce high levels of IL-10, moderate amounts of TGF-β and IFN-γ, but no IL-4 or IL-2. They exert immunoregulatory or suppressive effects.[215]

SUMMARY

Despite intensive research since Owen's first observations of immunologic tolerance in freemartin cattle, the mechanisms involved in spontaneous acceptance remain to be identified. Ultimately, the long-term goal of solid organ transplantation is induction of donor-specific tolerance to allow survival of the graft without the need for generalized immunosuppression. In addition, identification of the processes involved in the spontaneous acceptance of liver allografts will also advance our understanding of the complex immune system and potentially shed light on other immunologic processes.

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A NEW MODEL OF MOUSE ORTHOTOPIC LIVER TRANSPLANTATION WITH HEPATIC ARTERIALIZATION

INTRODUCTION

Orthotopic liver transplantation (OLT) remains the only definitive treatment for patients with end-stage liver disease. Although the one year success rate of liver transplantation currently exceeds 80%,[1] several questions remain to be answered regarding the mechanisms of acute and chronic graft rejection, organ preservation and reperfusion injury. Experimental OLT is a useful tool to study these questions. In 1973, Lee et al[2] described the first rat model of OLT with hepatic arterialization (A-OLT). To simplify the procedure, a second, non-arterialized model (nonA-OLT) was developed.[3] As a result, two models of rat OLT are currently used for organ transplantation research; one with dual blood flow from the hepatic artery and portal vein (A-OLT), and the second model supported solely by portal venous blood flow without hepatic arterialization (nonA-OLT). Later, Kamada et al[4] introduced the cuff technique for reanastomosis of the portal vein, infrahepatic vena cava and reconstruction of the common bile duct over a polyethylene stent. This method allowed shorter clamping time of the portal vein which resulted in improved survival.

The advantages of arterialization of rat liver grafts have been previously debated in the literature.[5,6,7,8,9,10,11] Proponents of the arterialized model argue that the absence of hepatic arterialization of liver grafts can lead to significant early mortality due to early graft loss secondary to common bile duct complications,[9,10] histological changes,[5,11] and varying immunologic reactions.[10,12] Furthermore, the A-OLT model represents a more physiological model.[7,8] Engemann and colleagues demonstrated in a rat model of syngeneic liver transplants that without re-arterialization of the graft there was fibrosis throughout the parenchyma and massive periportal cellular infiltration. This was in sharp contrast to the grafts that did undergo re-arterialization where there was no evidence of fibrosis or cells infiltrating the grafts.[10] In addition, Sumimoto and colleagues demonstrated that although arterialization of rat liver grafts may not be important for long-term survival,[13] they did see an increase in the serum levels of soluble donor MHC class I antigens and an increased number of inflammatory cells (mononuclear cells, macrophages and neutrophils) within the non-arterialized rat liver grafts.[12] Ischemia augmenting the allogeneic immune response may contribute to these differences. As a result, it would be difficult to separate the effects of non-specific immunologic responses from a specific allogenic graft response in studies of allograft rejection.

A mouse model of OLT offers significant advantages for immunologic research due to the well-defined mouse genome with numerous commercially available, genetically-defined inbred, transgenic, and knockout mouse strains. Genetic manipulation has resulted in mouse strains with unique immunologic characteristics, which permits the study of various aspects of the immune response. In addition, the availability of molecular probes, monoclonal antibodies, and reagents specific for the mouse species combined with decreased costs associated with purchasing and housing facilitate research using mouse models. At present, there has been no mouse model of A-OLT, to our knowledge, available to study the immunologic questions associated with graft rejection. The aim of this present study was to develop a viable mouse model of OLT with hepatic artery reconstruction. A technique of orthotopic liver transplantation in a mouse with reconstruction of the hepatic arterial supply to the graft is described.

MATERIALS AND METHODS

Animals. Male inbred syngeneic BALB/c mice (weighing 24-28g), purchased from the Health Sciences Laboratory Animal Services (HSLAS), University of Alberta, Edmonton, Canada, were used as size-matched donors and recipients of liver grafts. The mice were maintained in the HSLAS animal colony and all procedures were conducted in accordance with regulations set forth by HSLAS at the University of Alberta.

Surgical procedures. Under isoflorane inhalational anaesthesia and clean operating conditions, orthotopic liver transplantation was randomly performed using both arterialized (A-OLT) and non-arterialized (nonA-OLT) techniques with the assistance of an operating microscope. Our method for orthotopic liver transplantation was based upon techniques described by Lee,[3] Kamada,[4] and Qian.[5]

OLT without hepatic artery reconstruction (nonA-OLT)

Donor Operation. The liver was exposed through an abdominal transverse incision. Following gallbladder removal, the bile duct, portal vein and infrahepatic vena cava were identified and mobilized. The pyloric branch of the portal vein was ligated with an 8-0 silk suture and divided. The right adrenal vein was identified and ligated in a similar manner. To maintain an adequate length of the infrahepatic vena cava for cuff preparation, the right renal vein was ligated and divided close to the IVC. The suprahepatic vena cava was incised close to the diaphragm. A piece of polyethylene tubing (PE 10, outer diameter 0.61 mm, 2mm in length, Becton Dickinson, Parsippany, N.J., USA) was inserted into the lumen of the common bile duct. The aorta was mobilized and all of its branches (gastric artery, splenic artery, renal arteries, and superior mesenteric artery) were ligated and divided. The animal was then anticoagulated by injecting Heparin 30 IU diluted to 0.3ml intravenously into the penile vein. The liver was then flushed via a cannula in the portal vein with 0.4 ml lactated Ringer's solution at 4^{O}C. Immediately thereafter, the graft was placed in cold (4^{O}C) lactated Ringer's solution for cuff preparation until transplantation.

Cuff preparation. While immersed in cold lactated Ringers' solution, the cuffs were prepared from polyethylene tubing for the portal vein (PE 90, outer diameter 1.27 mm, Becton Dickinson, Parsippany, N.J., USA) and for the infrahepatic vena cava (PE 160, outer diameter 1.57 mm, Becton Dickinson, Parsippany, N.J.,USA). The procedures were facilitated by using a stabilizing clamp.

Recipient Operation. Through a transverse abdominal incision, the bile duct, hepatic artery and right adrenal vein were ligated and divided. The infrahepatic vena cava, portal vein and suprahepatic vena cava were cross-clamped. Once the recipient hepatectomy was completed, the graft was positioned. The suprahepatic IVC was anastomosed using a continuous 10-0 nylon suture (Dermalon, Davis & Geck Inc.) and the cuff technique[4] was utilized for anastomosis of the portal vein and infrahepatic IVC. Bile duct reconstruction was also performed using the cuff technique with a 2mm polyethylene stent (PE 10, outer diameter 0.61 mm, Becton Dickinson, Parsippany, N.J., USA) inserted into the lumen of the donor and recipient common bile ducts and secured with a circumferential 8-0 silk suture. The abdomen was closed in two layers using a running 5-0 absorbable suture.

Blood loss during surgery was replaced by warm lactated Ringer's solution, and body temperature was maintained with a heating blanket and intraperitoneal infusion of warmed fluid.

OLT with hepatic rearterialization (A-OLT)

Donor operation. Preparation of the portal vein, infrahepatic vena cava, and bile duct was similar to that in the nonA-OLT group. A segment of the aorta was carefully dissected free at the origin of the celiac artery to its junction with the hepatic artery (Figure 2-1). The infrahepatic and suprahepatic IVC and the portal vein were divided and the liver was excised.

Recipient operation. Implantation of the liver was similar to that in the nonA-OLT group. After venous revascularization and reconstruction of the bile duct, re-establishment of hepatic arterial circulation was performed by an end-to-side anastomosis of the donor aortic segment (with the hepatic artery and celiac axis) to the recipient infrarenal aorta using an 11-0 nylon running suture.[14]

Post-operative care and survival. Graft recipients were recovered in a warm nursery incubator for the first two post-operative days where they had access to food and water *ad libitum*. No antibiotics were given to the recipients. All animals that developed intraoperative complications (bleeding, thrombosis) and/or did not survive beyond the initial 24 hours were excluded from the study. It was assumed that these deaths were due to intra-operative technical complications. Liver recipients were observed daily for clinical evidence of bile duct obstruction. Recipients exhibiting lethargy, weight loss or jaundice were sacrificed; otherwise recipients in both A-OLT and nonA-OLT groups were followed until post-operative day 100 and then sacrificed using cervical dislocation under inhalational anesthesia. Complete necropsy was performed on all recipients at which time patency of the infra- and suprahepatic IVC, hepatic artery and portal vein anastomoses were assessed.

Histopathology. At autopsy, fresh graft tissue samples were taken for histopathological examination. Tissues were fixed in 10% buffered formalin solution, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

Statistics. All data are expressed as mean values ± standard deviations. Comparisons between groups were performed using the Fisher's exact test, Student's *t* test and Kaplan-Meier statistical analyses. P-values less than 0.05 were considered statistically significant.

RESULTS

A total of 76 mouse liver transplants were done; 37 A-OLT and 39 nonA-OLT. The first 35 grafts (15 A-OLT and 20 nonA-OLT) were performed to develop the arterialization techniques. The data presented here pertains to the subsequent 41 consecutive transplants surviving greater than 24 hours following transplantation.

Operative time

The portal vein and infrahepatic IVC clamp times were similar in both A-OLT and nonA-OLT groups (Table 2-1). The total operative time, which includes both the donor and recipient procedures, however, was significantly longer in A-OLT group compared to the nonA-OLT group (135.4 min *versus* 76.7 min; $p<0.001$) due to the time required to isolate and reconstruct the hepatic arterial supply to the graft in the A-OLT group (Table 2-1). However, the total portal vein clamp time was similar between groups (13.7 min vs 14.0 min). Liver graft recipients received an average of 0.3 ml and 0.6 ml of lactated Ringer's solution intravenously as fluid replacement in the nonA-OLT and A-OLT groups, respectively.

Survival rate

To calculate survival rate, animals that died during the first 24 hours following transplantation were excluded from the study since it was assumed that these deaths were due to operative and/or anaesthetic misadventure. In the A-OLT group, the survival rate at post-operative day 14 was 87.0% compared to 52.6% in the nonA-OLT group (Figure 2-2). Furthermore, the survival rate at post-operative day 100 was significantly higher in the A-OLT group compared to the nonA-OLT group (56.5% *versus* 31.6%, $p=0.03$). All deaths in the nonA-OLT group occurred by day 27 where the rate of survival declined to 31.6% compared to 86.4% in the A-OLT group. Autopsy and subsequent histological analysis demonstrated that the major cause of death in the nonA-OLT group was common bile duct necrosis with bile leakage (n=7/19). In contrast, only 1 bile duct necrosis with subsequent bile leak occurred in the A-OLT group.

Surgical complications

Bile duct necrosis with biliary leakage.

The incidence of biliary leakage was significantly higher in the nonA-OLT group than in the A-OLT group (p=0.02). Only one of 22 recipients (4.5%) in the A-OLT group developed bile duct leakage. This recipient had a long aberrant hepatic artery that was found to be thrombosed on autopsy. In contrast, in the nonA-OLT group, 7 of 19 (36.8%) recipients had evidence of biliary leakage. These animals developed jaundice and thus, were sacrificed or found dead between days 4 and 12 following transplantation. Autopsy revealed local biliary peritonitis with partial liver necrosis in the majority of cases.

Common bile duct obstruction.

Common bile duct obstruction was a typical observation in the later stages following transplantation in both the nonA-OLT and A-OLT groups. Four of 22 recipients (18.2%) in the A-OLT group developed jaundice at various times following transplantation ranging from post-operative day 17 to 44, and were subsequently sacrificed. In most cases, autopsy revealed dilatation of the common bile duct proximal to the site of bile duct reconstruction. The causes of common bile duct obstruction included migration of the bile duct stent in two A-OLT recipients and a third recipient had a rotation in the reconstructed bile duct. The fourth A-OLT recipient with a common bile duct complication had no evidence to suggest an explanation for the bile duct dilatation. The arterial and venous anastomoses were patent in all four recipients. In contrast, two of 19 recipients (10.5%) in the nonA-OLT group had common bile duct obstruction. These two mice were jaundiced by 2 weeks following transplantation. Of these recipients, one had a rotation of the reconstructed common bile duct and the other recipient had migration of the bile duct stent.

Other complications

Hypovolemic shock resulted in the early death of 1 of 22 recipients (4.5%) in the A-OLT group and one of 19 recipients (5.3%) in the nonA-OLT group. The main site of blood loss in these two animals was from a liver injury during the time of transplantation. One recipient in the A-OLT group became lethargic with declining body weight and thus, sacrificed on post-operative day 12. Postmortem examination demonstrated that this mouse had multiple hepatic abscesses with a normal common bile duct and patent vascular anastomoses. Portal vein thrombosis also resulted in the death of a recipient in the A-OLT group that died on post-operative day 31. Venous thrombosis (one in the suprahepatic and the other in the infrahepatic vena cava) led to early deaths in two recipients in the nonA-OLT group.

Histopathology

Histological examination of the liver grafts harvested from long-term survivors (> 100 post-operative days) in the A-OLT group revealed normal architecture with normal size and structure of portal tracts (Figure 2-3A). There was no evidence of bile duct proliferation and very few inflammatory cells infiltrating the A-OLT grafts in long-term survivors. Grafts harvested from long-term survivors in the nonA-OLT, however, demonstrated neutrophilic cellular infiltration of the portal triads, endothelialitis and significant bile duct proliferation (Figure 2-3B).

DISCUSSION

Although previous authors have reported excellent survival rates in their murine model of nonA-OLT, several complications due to lack of arterialization have been reported in other OLT models including decreased survival, bile duct complications, microcirculatory disorders and histological changes. Furthermore, although previous studies have demonstrated that arterialization of rat liver grafts may not be important for long-term survival,[15] an increase in the serum levels of soluble donor MHC class I antigens and an increased number of inflammatory cells (mononuclear cells, macrophages and neutrophils) are present within the non-arterialized rat liver grafts;[12] likely the result of ischemia augmenting the allogeneic immune response. As a result, it would be difficult to separate the effects of non-specific immunologic responses from a specific allogeneic graft response in studies of allograft rejection using a non-arterialized model. Therefore, our objective was to develop a robust model of liver transplantation in a mouse to study the mechanisms mediating acute and chronic allograft rejection, organ preservation and reperfusion injury. The value of hepatic arterialization in a mouse model of OLT has not been established prior to this study.

Biliary drainage has long been recognized as one of the most difficult problems compromising the success of clinical liver transplantation. Several factors may contribute to biliary complications including inadequate arterial blood supply to the bile duct, poor operative technique and graft rejection. The biliary tract is supplied and nourished by a network of fine vessels called the peribiliary vascular plexus which is fed by afferent vessels from the interlobular hepatic artery.[16] This distinct and highly complex peribiliary vascular structure renders the bile duct particularly vulnerable in situations of devascularization. Thus, partial or complete loss of arterial blood supply, could lead to bile duct necrosis with biliary leakage as evidenced in the nonA-OLT group. Without hepatic arterialization, the incidence of biliary leakage secondary to bile duct necrosis in the nonA-OLT group was significantly higher than in the A-OLT group. In our study, re-arterialization almost completely eliminated bile duct leakage in the A-OLT group. Therefore, as suggested by previous investigators,[8,9] hepatic arterial blood flow is crucial for maintaining competence of the biliary tract, and lack of arterialization may result in a high incidence of biliary leakage due to bile duct necrosis in a mouse model of OLT.

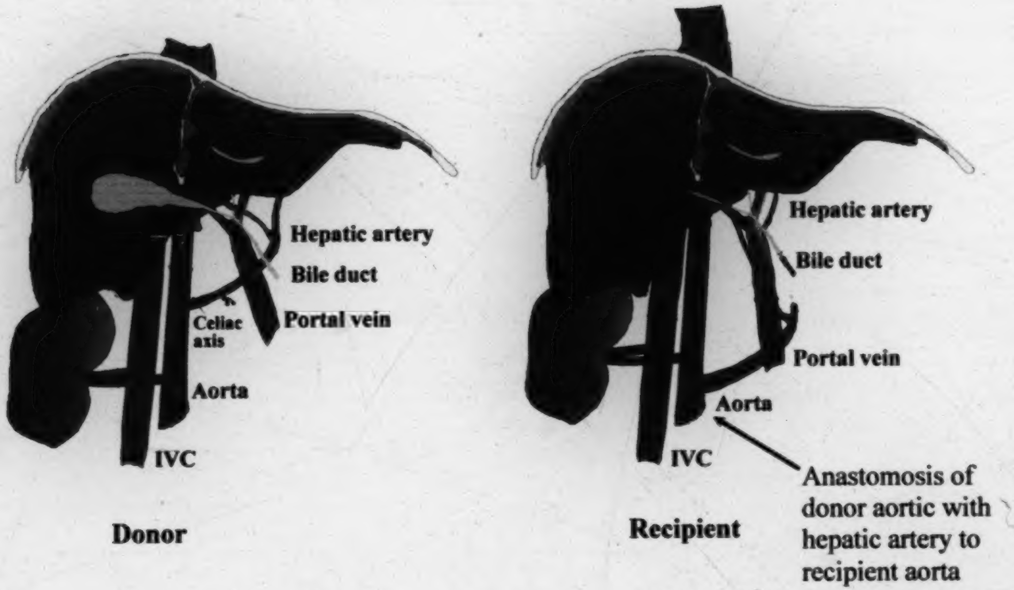
Our current study revealed that the majority of deaths were due to the biliary complications of duct necrosis with bile leakage or common bile duct obstruction. The former complication represented the main factor determining early survival, and the latter affected late survival. The higher incidence of bile duct necrosis and leakage (42.1%) resulted in a significant decrease in early (54.6%) and overall survival (31.6%) in the nonA-OLT group. The A-OLT group had a much lower incidence of bile duct leakage (4.5%) which translated into improved early survival rate (86.4%). Therefore, hepatic rearterialization appears to have the greatest impact on early graft survival. Thus, perfusion injury (early graft studies) and graft rejection (early and long-term graft studies) are best studied by an A-OLT model which has the greatest early and long-term graft survival.

Common bile duct obstruction was the major cause of late deaths in both groups. All episodes of obstruction occurred above the implanted stent, which may indicate that the non-absorbable polyethylene tubing plays a pathogenic role in common bile duct obstruction by inciting a “foreign body” inflammatory reaction and/or bile sludging.[17] Thus, the anastomotic site of the bile duct may be either occluded or constricted and subsequently a complete or partial bile duct obstruction may develop. In some cases the common bile duct obstruction was secondary to a mechanical obstruction such as migration and/or malrotation of the bile duct stent or a rotation of the bile duct itself. The use of a short stent (<2 mm) in our experiments might have been the cause for the bile stent migration observed in some recipients[5] and we have since switched to a longer length (4 mm) which has eliminated this complication. A stent manufactured from bioabsorbable material may be a way to prevent these technical complications.

The histology of the graft was also affected by arterial supply to the graft. Long-term survivors with non-arterialized syngeneic grafts demonstrated evidence of non-specific inflammatory changes within the graft including cellular infiltrates, fibrosis within the portal triads and bile duct proliferation. These histological changes within the graft can not be easily distinguished from immunologic changes that occur during acute and chronic allograft rejection episodes. In contrast, examination of grafts with hepatic arterialization demonstrated normal architecture of the portal triads. Thus, hepatic re-arterialization of the graft reduced nonspecific inflammatory histologic changes in the graft related to the procedure itself and will allow greater clarity in interpreting immunologically-mediated changes in future studies of allograft rejection.

Due to its small blood volume, the mouse is much less tolerant of hypovolemic shock, which can be caused by blood loss, prolonged portal vein clamping time and insufficient crystalloid solution replenishment. These factors are likely to account for the early (<24hrs) death of mice in our study. In addition, mouse OLT with re-arterialization can be further complicated by blood loss during the dissection of the aortic segment, prolonged operative time, and the increased technical skill required for anastomosis of the hepatic artery and thus, may be associated with a higher early mortality. In our current study, however, deaths due to hypovolemic shock and/or arterial anastomosis failure occurred in a small number of recipients in the A-OLT group. The total operative time was longer in the A-OLT group compared to the nonA-OLT group due the additional time required to harvest and reconstruct the arterial supply to the graft. Portal vein cross-clamping time[4,5] is one of the most important factors for successful OLT such that prolonged portal vein clamping periods (>20 minutes) can be lethal by compromising the portal venous and systemic circulation systems resulting in irreversible hypovolemic shock. Our study demonstrated that in our hands, portal vein cross-clamping time was markedly less than 20 minutes in both nonA-OLT and A-OLT groups (Table 2-1) and was not different between the two study groups.

In summary, we have successfully developed a new surgical model of mouse orthotopic liver transplantation with hepatic arterialization. The data presented in this report supports the concept that hepatic arterial supply is important in preventing biliary ischemia with resultant complications such as common bile duct obstruction or necrosis with bile leakage in mouse liver transplantation. Hepatic arterialization prevents early graft loss secondary to common bile duct complications and therefore, increases early and overall graft survival compared to a non-arterialized model. In addition, an arterialized mouse model represents the ideal model for studies of graft rejection and preservation injury since nonspecific inflammatory changes that occur in the absence of arterialization may confound histologic and cellular analysis of the graft when attempting to identify the mechanisms involved in rejection. The results from our study have important implications for future research in liver transplantation.


Hepatic artery
Bile duct
Celiac axis
Portal vein
Aorta
IVC
Hepatic artery
Bile duct
Portal vein
Aorta
IVC

Anastomosis of donor aortic with hepatic artery to recipient aorta

Donor

Recipient

Figure 2-1. Orthotopic liver transplantation with hepatic re-arterialization. A long segment of the aorta with its celiac axis and hepatic artery is harvested and used to reconstruct the arterial supply to the liver graft in the recipient.

Survival of Arterialized Versus Non-arterialized Syngeneic Liver Grafts

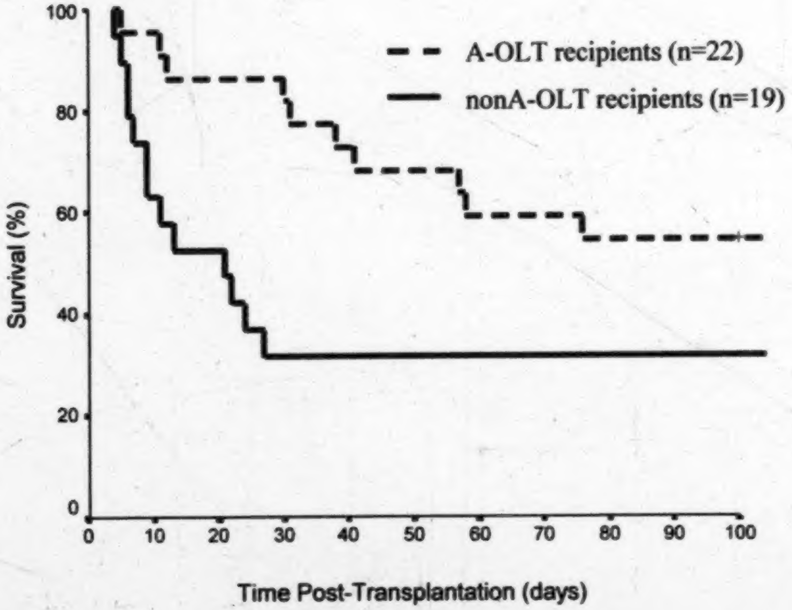


Figure 2-2. Survival of Arterialized Versus Non-arterialized Syngeneic Liver Grafts. Overall survival at day 100 following transplantation was significantly higher in the A-OLT group than in the nonA-OLT group (54.6% versus 31.6%; p=0.03).

Table 2-1. Operative times of mouse liver transplantation with (A-OLT) or without hepatic rearterialization (nonA-OLT).

	A-OLT (n=22) (min)	nonA-OLT (n=19) (min)	P value
Donor Procedure (mean ± SD)	55.4 ± 4.4	28.5 ± 3.3	<0.001
Portal Vein Clamp Time (mean ± SD)	13.7 ± 1.4	14 ± 1.5	NS[a]
Infrahepatic IVC Clamp Time (mean ± SD)	15.6 ± 1.5	15.2 ±1.1	NS[a]
Recipient Procedure (mean ± SD)	80 ± 4	48.2 ± 4	<0.001
Rearterialization Time (mean ± SD)	43 ± 3.5	-	
Total Operative Time (mean)	135.4	76.7	<0.001
IV fluids	0.6 ml	0.3 ml	

[a] NS; not statistically significant

Table 2-2. Complications leading to death following mouse OLT transplantation.

	A-OLT (n=22)	NonA-OLT (n=19)	P
Common bile duct obstruction	4 (18.2%)	2 (10.5%)	NS
Biliary leakage	1 (4.5%)	7 (36.8%)	0.02
Hypovolemic shock	1 (4.5%)	1 (5.3%)	NS[a]
Venous thrombosis	1 (4.5%)	2 (10.5%)	NS[a]
Liver abscesses	1 (4.5%)	0	NS[a]
Total	8 (36.4%)	12 (63.2%)	NS[a]

[a] NS; not statistically significant

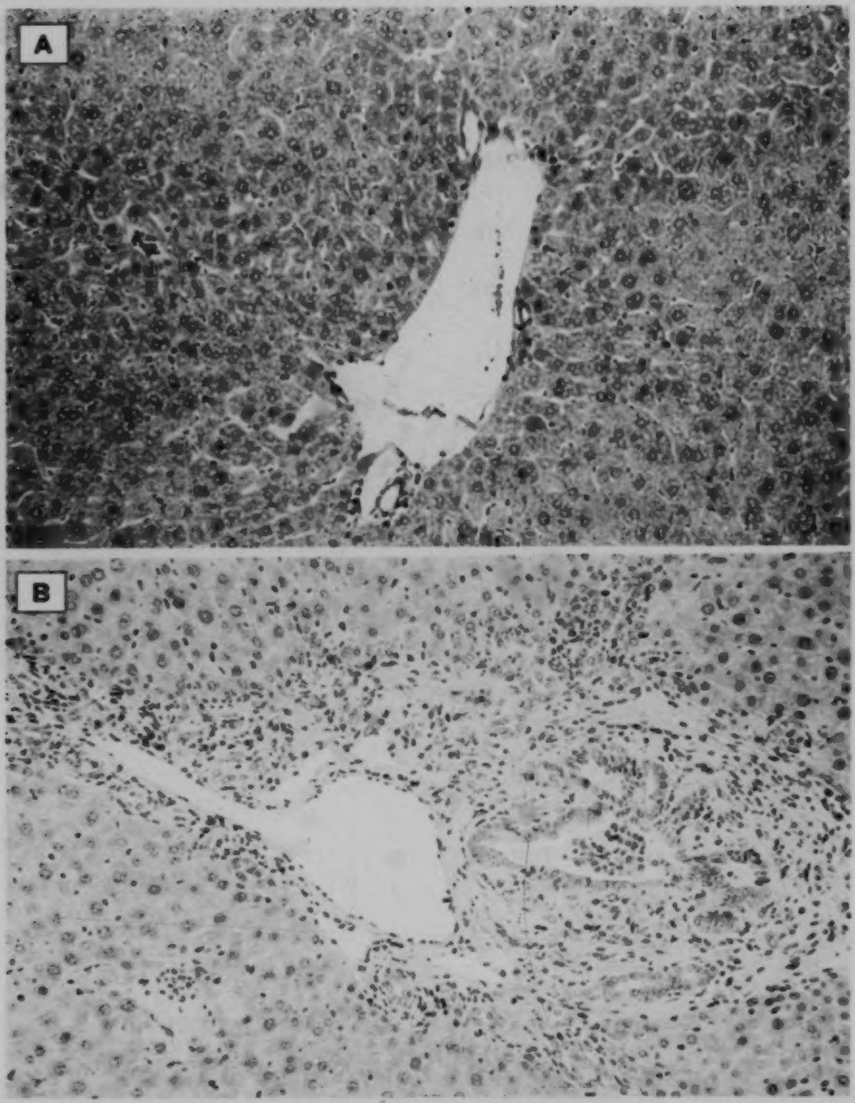


Figure 2-3. Histology of syngeneic liver grafts harvested from mice surviving long-term (> 100 days). (A) Arterialized graft demonstrating normal architecture with minimal cellular infiltration or bile duct proliferation (H&E; original magnification x 100). (B) Non-arterialized graft demonstrating extensive neutrophilic infiltration and bile duct proliferation (H&E; original magnification x 100).

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IFN-γ IS AN ABSOLUTE REQUIREMENT FOR SPONTANEOUS ACCEPTANCE OF LIVER ALLOGRAFTS

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INTRODUCTION

Spontaneous acceptance of MHC-disparate liver transplants in the absence of immunosuppressive therapy is a striking observation in many experimental liver transplant models(1-3). Furthermore, spontaneously accepted liver allografts may induce acceptance of other organs including heart or kidney allografts without the need for immunosuppression(2,4). The mechanisms mediating spontaneous acceptance of livers and the ability of liver transplants to modify rejection of other transplants has implications for understanding the mechanisms regulating graft rejection and host-graft adaptation in general. Among the unique features of the liver as a transplant is its mass in relationship to a finite immune response. Spontaneous acceptance of other organs can occur when multiple organs are transplanted into one host(5). However, other features of the liver probably play a role, including its remarkable capability to regenerate after loss of mass, the properties of the portal circulation, and possible tolerogenic properties of liver antigen presenting cells(6).

One factor that could influence spontaneous acceptance is IFN-γ, which acts via a specific receptor (IFN-γR)(7) to exert numerous effects in transplantation. These include direct effects on the graft such as induction of donor MHC expression(8), direct effects on the host alloimmune response including limitation of host CTL generation(9,10), and induction of systemic MHC expression in the host(11,12). In the early post transplant period, a major effect of IFN-γ in rejecting heart(9,10,13) and kidney transplants(14) is to protect against necrosis by an unknown mechanism, despite inducing high MHC expression(9). Thus kidney transplants lacking IFN-γ receptors develop necrosis and congestion, indicating that IFN-γ acting directly on the receptors of the allograft protects against failure of the microcirculation during acute rejection(15). Transcription factor IRF-1 is necessary for this protective effect(16). In addition, IFN-γ is necessary for initiating rejection of MHC class II-disparate skin allografts(17), perhaps because it must induce donor MHC class II for rejection to occur. The absence of IFN-γ accelerates heart graft rejection in mice lacking IL-2(18), and a suppressor role for IFN-γ has been demonstrated in rejection of rat heart transplants(19).

How these effects of IFN-γ would operate in liver transplantation is unknown, and of considerable interest given the unique features of the liver. In the present studies we examined the systemic and local effects of IFN-γ during the spontaneous acceptance of liver allografts, using hosts deficient in IFN-γ production or grafts lacking IFN-γRs. Our results indicate that IFN-γ is absolutely required for spontaneous acceptance and for MHC induction in liver transplantation, acting at least in part by a direct effect on the liver itself.

MATERIALS AND METHODS

Mice

BALB/c (H-2^d), CBA/J (H-2^k) and 129/SvJ mice (H-2^b) were purchased from Jackson Laboratories, Bar Harbor, ME. IFN-γ-deficient mice were generated as described previously(20). Briefly, a normal allele in mouse embryonic cells was replaced with a defective gene using a targeted vector which introduced a termination codon after the 30 first amino acids of the mature IFN-γ protein. These stem cells were used to construct mice heterozygous for the disrupted gene, which were intercrossed and the progeny were selected for homozygosity. Breeding pairs of IFN-γ deficient mice with disrupted IFN-γ genes (GKO mice, H-2^d on a BALB/c background) were a generous gift from Dr. Tim Stewart (Genentech Inc., South San Franscisco, CA) and a colony is maintained at HSLAS, University of Alberta, Edmonton, Canada. The original GRKO (129/Sv/Ev, H-2^b) mouse strain with disrupted IFN-γR α-chain genes was generated by gene targeting in murine embryonic stem cells(21). The gene was disrupted by inserting the neomycin-resistance gene into exon V, which encodes an extracellular domain. Homozygous 129/Sv/Ev mice were kindly provided to us through Dr. Michael Aguet (University of Zurich, Zurich, Switzerland) and a breeding colony is housed in the HSLAS facility at the University of Alberta, Edmonton, Canada. All procedures were undertaken in accordance with Animal Care Protocols from HSLAS at the University of Alberta which are in accordance with the Canadian Committee on Animal Care.

Orthotopic Liver Transplants

Livers from male mouse donors were isolated and transplanted into male mice weighing 25-30 g according to a newly developed model of murine orthotopic liver transplantation with hepatic arterialization. IFN-γ knockout mice (GKO, H-2^d) and BALB/c (H-2^d) were transplanted with CBA/J (H-2^k) livers. The IFN-γ receptor knockout (GRKO, H-2^b) and 129/SvJ H-2^b) livers were used as donors for CBA/J (H-2^k) recipients. For syngenic transplants, BALB/c livers were transplanted into BALB/c and GKO livers were transplanted into GKO. Briefly, under isofluorane anesthesia, a transverse abdominal incision was followed by dissection and division of the portal and supra-hepatic veins, hepatic artery and common bile duct and removal of the liver. The host mice were similarly anaesthetized and the donor liver implanted orthotopically with reanastomosis of the portal and supra-hepatic veins and hepatic artery. The common bile duct was reconstructed over a polyethylene stent. No immunosuppressive therapy was administered at any time during the experiments. Mice receiving liver grafts were monitored daily for evidence of graft failure (jaundice, decreased activity and weight loss). Graft loss due to rejection was confirmed by histologic examination of tissues obtained at autopsy. Spontaneous acceptance was defined as liver allograft survival at 100 days following transplantation in the absence of immunosuppressive drug therapy. Statistical analysis of spontaneous acceptance rates in each of the strain combinations was performed using Kaplan-Meier statistical analysis (SPSS, Chicago, Ill.).

Evaluation of donor-specific tolerance

Mice with liver allografts surviving >100 days were transplanted with syngeneic (BALB/c, H-2^d), donor-specific allogeneic (CBA/J, H-2^k) and third-party allogeneic (129/SvJ, H-2^b) skin grafts at the same time(22). Briefly, a full-thickness graft bed was prepared by surgically excising a 0.5 cm x 0.5 cm section of skin from the dorsum of each skin-graft recipient and removing all subcutaneous tissue to the deep fascia. A similar full-thickness skin graft was harvested from the dorsum of each skin-graft donor, placed on the graft bed and sutured in place with four 8-0 prolene sutures and secured with gauze and adhesive tape to prevent shearing. The protective covering was removed at day 3 to allow monitoring for evidence of rejection. Grafts were scored as rejected when viable tissue could no longer be detected. Rejection or acceptance of skin grafts was confirmed by excision and histologic examination.

Histopathology

At autopsy, fresh tissue samples were fixed in 10% buffered formaldehyde, paraffin-embedded, sectioned and stained with hematoxylin and eosin. Pathological assessment of liver sections was conducted by a pathologist (L.J.) who was blinded to the strain combinations used in the various experiments. Severity of liver allograft rejection was determined using the Banff grading system(23) which assigns cellular infiltration of portal triads, bile duct inflammation and endothelial inflammation a score of 1-3: 1, minimal changes; 2, moderate changes; or 3, severe changes. Additional findings not included in the Banff scoring system were also studied. The extent of necrosis was scored as the percentage of parenchymal involvement (0, no necrosis present; 1, <25% of the total parenchyma involved; 2, 25-50% of total parenchyma involved; 3, 50-75% of the total parenchyma involved and 4, >75% of total parencyma involved). Mann-Whitney statistical analysis was used to compare the severity of histologic lesions seen in CBA grafts transplanted into WT mice versus GKO mice.

Antibodies

Hybridoma cell lines producing mouse monoclonal antibodies (mAb) 34-4-20S (anti H-$2D^d$), 25-9-17SII (anti I-A^d), 11-4.1 (K^k), 11-5.2.1.9 (I-A^k); 20-8-4S (K^bD^b) and AF6-120.1.2 (I-A^b); and rat monoclonals M1/42.3.9.8 (anti H-2 antigens, all haplotypes) and M5/114.15.2 (anti I-$A^{b,d,q}$ and I-$E^{d,k}$) were obtained from the American Tissue Culture

Collection (Manassas, VA). Supernatants containing mouse monoclonals were purified by protein A chromatography. The supernatants from M1/42.3.9.8 and M5/114.15.2 cell lines were precipitated using ammonium sulphate, purified through a DE52 anion exchanger column (Whatman, Hillsboro, OR), and concentrated by Amicon ultrafiltration (Beverly, MA). The protein concentration was determined by a modified Lowry method, adjusted to 1 mg/ml and kept frozen at –70 °C.

Radiolabelled antibody binding assay (RABA)

A radiolabelled antibody-binding assay was used to quantify MHC class I and II expression using allospecific mouse mAbs against MHC class I and II of H-2^d (WT and GKO BALB/c), H-2^k (CBA/J) and H-2^b (WT and GRKO 129/SvJ) haplotypes. This technique has been previously reported(24) and its semi-quantitative characteristics have been previously described(25,26). Monoclonals anti-H-$2D^d$, anti-I-A^d, anti K^k, and anti-I-A^k mAb, anti-H-$2K^bD^b$ mAb, and anti-I-A^b mAb were radiolabelled with [125I] iodide using the Iodogen method (Pierce, Rockford, IL)(27). Briefly, liver graft tissue obtained at various times following transplant was frozen in liquid nitrogen and stored at –70 °C. Liver tissue of individual mice was homogenized in 1 ml PBS, washed in 10 ml PBS, and centrifuged at 3000 rpm for 20 minutes. The pellets were suspended in PBS at a concentration of 10mg/ml. Liver tissue (2.5mg) was centrifuged and resuspended in 100μL of radiolabelled mAb (100,000 cpm) and incubated on ice with agitation for 60 min. One milliliter of PBS was added to all of the tubes and spun at 3000rpm for 20 min. The pellets were counted in a gamma counter and the nonspecific binding of a control negative tissue was subtracted. The results are expressed as specific cpm bound by the tissue homogenate after subtracting the background cpm absorbed by the negative control tissue. Based on standard curves, a two-fold change in specific cpm bound in this assay corresponds to approximately a three-fold change in antigen output(28). However the results of such assays must be considered semi-quantitative(29).

Indirect Immunoperoxidase Staining

The pattern of MHC expression was determined by indirect immunoperoxidase staining(26). Briefly, 4 µm-thick sections were cut from frozen liver allograft samples embedded in Tissue-Tek OCT (Skura Finetek, Torrance, California) and mounted on poly-l-lysine-coated glass microscope slides, fixed in acetone, and then incubated with normal goat serum. The slides were then incubated with rat anti-mouse MHC class I (M1), class II (M5), mouse MHC class I H-2D (host) and H-2K (donor) and class II I-A^d and I-A^k monoclonal antibodies or phosphate-buffered saline (PBS) as a control. The slides were then incubated with affinity-purified peroxidase-labeled goat anti-rat or goat anti mouse IgG $F(ab')_2$ fragment (ICN, Costa Mesa, CA). The slides were then incubated with 3'3 diaminobenzidine tetrahydrochloride and hydrogen peroxide for a color reaction and counterstained with hematoxylin. Previous studies in our laboratory have not shown differences between staining obtained with PBS and isotype controls, both of which are negative when using rat anti mouse monoclonals M1 and M5. MHC staining was also confirmed by host specific and donor specific mouse monoclonals class I H-2D (host) and H-2K (donor) and class II I-A^d and I-A^k and goat anti-mouse second antibody.

Assessment of gene expression

Granzyme A, granzyme B, perforin, Fas ligand (FasL), and hypoxanthine phosphoribosyltransferase (HPRT) expression were assessed by TaqMan real-time PCR. Total RNA was extracted from individual liver graft tissue obtained at various times post-transplant. Briefly, liver tissue, stored in liquid nitrogen at –70°C, was homogenized in 4M guanidinium isothiocyanate, and the RNA was pelleted through a 5.7M $CsCl_2$ cushion. RNA was transcribed into cDNA and amplified in ABI Prism 7700 sequence detection system using sequence specific primers and probes listed in Table 3-1. The cDNA was amplified in a multiplex system using HPRT as the control gene. The data was analyzed using the sequence detector software (Applied Biosystems, Foster City, CA.)

RESULTS

Spontaneous acceptance of liver transplants and the role of IFN-γ.

We transplanted syngeneic and allogeneic livers into WT and GKO hosts. Long-term survival of syngeneic BALB/c livers transplanted into BALB/c mice (n=20) was 45% at 100 days post-transplant (Figure 3-1). The majority of graft loss in these syngeneic transplants was due to common bile duct complications. Thus during the early post-transplant period, two mice died of bile duct leaks (day 5 and day 11), and a third mouse died at day 12 due to multiple liver abscesses. Later in the post-transplant period, most grafts were lost due to biliary duct obstruction secondary to ischemia resulting in biliary duct stenosis. When allogeneic CBA livers were transplanted into WT mice (n=33) the survival rate was 42.4% at 100 days post-transplant, which was not statistically different from syngeneic transplants (p=0.74). Thus in WT hosts few if any grafts were lost to rejection, beyond the rate expected from technical difficulties as observed in the control syngeneic transplants.

In contrast, no CBA liver allograft transplanted into a GKO mouse survived longer than 14 days (n=15) (Figure 3-1, p<0.0001 CBA allografts into WT versus GKO mice). Most of these failures occurred between day 7 and day 14. To determine if the loss of liver allografts in GKO hosts was immunologic, we transplanted syngeneic GKO livers into GKO hosts. All syngeneic GKO grafts survived beyond 100 days (n=4).

We transplanted syngeneic (BALB/c), donor-specific (CBA) and third-party (129/SvJ) skin grafts onto hosts with spontaneously accepted liver allografts to evaluate the specificity of liver allograft tolerance. Both syngeneic (BALB/c, $H\text{-}2^d$) and donor-specific allogeneic (CBA/J, $H\text{-}2^k$) skin grafts were accepted and survived long-term (> 100 days) (n=7), whereas allogeneic third-party (129/SvJ, $H\text{-}2^b$) skin grafts were rejected by all mice which had spontaneously accepted liver allografts.

Histologic analysis of rejecting liver allografts

Using a modification of the Banff grading system for liver allograft rejection, we observed higher scores for inflammation in grafts harvested from GKO mice ($H\text{-}2^d$) compared to WT mice (BALB/c, $H\text{-}2^d$) (Table 3-2) (Figure 3-2). At days 5, 7 and 10 post-transplant, CBA ($H\text{-}2^k$) liver allografts in GKO mice manifested greater infiltration of portal triads, inflammation of veins, and invasion of bile duct epithelium by lymphocytes. There was no hemorrhage or parenchymal necrosis in WT recipients (Figure 3-2A, B) but there was extensive necrosis with some congestion and hemorrhage in livers rejecting in GKO hosts (Figure 3-2C, D). The parenchymal necrosis was concentrated in zones 2 and 3 of the hepatic acinus, with sparing of zone 1, the peri-portal areas. This pattern indicates ischemic necrosis.

Liver allografts in WT hosts surviving > 100 days following transplantation demonstrated resolution of the inflammation, leaving only foci of mononuclear cells with no necrosis or hemorrhage (Figure 3-2E). There was no disturbance of the portal triad architecture.

Evaluation of the direct effects of IFN-γ on liver allografts

We studied whether in protecting the liver against necrosis the IFN-γ was acting on the graft or the host. We compared rejecting liver allografts from mice lacking IFN-γ receptors (GRKO, H-2^b) to those from wild-type mice with intact IFN-γ receptors (129/SvJ, H-2^b) that were transplanted in CBA hosts (H-2^k). We hypothesized that if the protective effects of IFN-γ are due to regulation of the host immune response, then the pattern of acceptance of grafts lacking IFN-γ receptors should be similar to that of WT grafts. Conversely, if the effect is on the liver transplant itself, then the grafts from donors lacking IFN-γ receptors should manifest increased destruction resembling that in GKO hosts.

Wild-type (129/SvJ) allografts demonstrated infiltration typical of acute rejection, with no necrosis at day 10 post-transplant (Figure 3-2F). GRKO liver allografts at day 10 post-transplant demonstrated increased cellular infiltrate with necrosis of the parenchyma (Figure 3-2G and H). The grading of the pathology is shown in Table 3-3. Thus the phenotype of rejecting allografts lacking IFN-γ receptors resembled that of allografts transplanted into hosts lacking IFN-γ, with greatly increased necrosis. Unlike the latter, there was less increase in the infiltrating inflammatory cells in portal triads in the GRKO livers compared to the WT livers.

MHC expression in liver allografts

We measured donor MHC expression in the grafts using a radiolabeled antibody-binding assay described previously (30). CBA liver grafts transplanted into WT hosts showed induction of donor (H-2^k) MHC class I expression (Figure 3-3), with more variable induction of donor class II expression. In contrast, neither class I or class II was induced in liver grafts transplanted into GKO hosts.

We confirmed these differences in MHC expression by indirect immunoperoxidase staining, using rat monoclonal antibodies specific for mouse MHC class I and II. In the control sections from rejecting transplants, peroxidase-positive cells were observed in the infiltrate in the rejecting transplants, both in WT and GKO hosts (Figure 3-4A and 4B). MHC class I was increased on the endothelial cells and sinusoidal cells of grafts into WT hosts, as well as on the cellular infiltrate (Figure 3-4C). In contrast, there was little staining for MHC class I in the endothelial or parenchymal cells or the infiltrating cells in grafts transplanted into GKO hosts (Figure 3-4D).

Class II expression in normal mouse liver is confined to occasional interstitial cells called dendritic cells(11,31,32). Class II was increased in livers rejecting in WT hosts (Figure 3-4E) in discreet positive cells in sinusoids, which have previously been shown to be Kupffer cells(31). These were absent in liver transplants in GKO hosts, whose class II expression remained in the basal state (Figure 3-4F).

The MHC staining was confirmed using donor-specific mouse monoclonals anti class I (K^k) and anti class II mouse monoclonals ($I\text{-}A^k$). Again the livers rejecting in BALB/c hosts showed diffuse increased staining of endothelium, Kupffer cells, and the sinusoidal face of the hepatocytes for class I; and increased staining of individual Kupffer cells for class II. This increased staining was absent in GKO hosts, whose MHC staining remained in the basal state. Host specific monoclonals against D^d and $I\text{-}A^d$ showed staining only of the infiltrating cells in the rejecting livers in BALB/c mice but much less staining of the infiltrate in GKO mice.

For the 129 WT liver allografts in CBA hosts, the RABA demonstrated strong induction of donor MHC class I and moderate induction of donor class II antigens (Figure 3-5). In contrast, GRKO grafts had no induction of donor MHC class I or class II expression, remaining at basal expression.

CTL gene expression

We compared expression of a number of genes associated with CTL activity (granzyme A and B, FasL and perforin) in rejecting liver allografts in WT and GKO mice, at days 5, 7 and 10 post transplant by Real-time RT-PCR analysis (Figure 3-6). Expression of all of these genes was massively elevated post-transplant in grafts transplanted into WT or GKO hosts, peaking at day 5 and declining at days 7 and 10. However, the levels of granzyme A, granzyme B, and Fas L mRNA were significantly lower in GKO hosts at days 5 and 7. The differences were too great to be attributable to the necrosis in the liver allografts in GKO hosts, and were surprising in view of the heavier infiltrate of lymphocytes in the GKO transplants. Perforin mRNA levels were similar in grafts transplanted into WT and GKO hosts.

DISCUSSION

These studies of liver allograft rejection in hosts lacking IFN-γ and in normal hosts with livers from donors lacking IFN-γ receptors indicate that IFN-γ action on the graft is an absolute requirement for the spontaneous acceptance of liver allografts. There were no survivors if IFN-γ was not present, whereas if IFN-γ was present the survival of MHC incompatible livers was similar to that of the syngeneic controls i.e. complete spontaneous acceptance. Comparison of liver rejection in WT and GKO hosts establishes that IFN-γ affected several aspects of the early rejection phenotype: it reduces hemorrhage, necrosis and cellular infiltration while inducing high MHC expression. Despite the aggressive rejection in GKO hosts, mRNAs for genes associated with T cell effector activity were not increased in the GKO recipients. The phenotype of rejecting liver allografts lacking IFN-γ receptors was similar in that parenchymal necrosis was increased and MHC expression was not induced. The necrosis was dependent on the alloimmune response, coinciding in time with rejection and being absent in syngeneic grafts. The results point to a major effect of IFN-γ being on the graft itself, inducing donor MHC expression and preventing ischemic necrosis, while leaving open the likelihood of significant direct effects on host immune regulation.

The ability of IFN-γ to prevent graft necrosis during rejection via graft IFN-γ receptors is observed in several types of vascularized organ allografts. Excessive necrosis has been observed in kidney allografts in GKO hosts(30) and in kidneys from donors lacking the IFN-γRs(15). This effect was also seen in kidneys lacking transcription factor IRF-1(33), suggesting that this IFN-γ-regulated transcription factor acts distally to IFN-γ receptors.(15,33) A potentially related effect has been reported in concordant xenografts when the host lacks IFN-γ (14). IFN-γ does not act across unrelated species, suggesting that lack of host IFN-γ action on the graft may contribute to the vascular destruction in discordant xenografts. However, administration of recombinant IFN-γ to GKO hosts may not readily prevent necrosis, as shown in our earlier studies(30). This may reflect the fact that production of IFN-γ in the rejecting graft is massive and possibly paracrine, acting on contiguous cells, and may be difficult to simulate by systemic IFN-γ administration. We have also attempted to prevent liver allograft necrosis by injecting rIFN-γ intraperitoneally in GKO recipients but as yet with limited success (unpublished results). We investigated this in our kidney model and found that the administered anti IFN-γ was not capable of neutralizing IFN-γ produced in the graft. Others have also found that anti IFN-γ failed to alter survival.

The destruction of rejecting livers in IFN-γ deficient hosts resembled ischemic necrosis despite patent vessels, suggesting failure of the microcirculation. This suggests that IFN-γ sustains the microcirculation, by an action that requires IFN-γ receptors in the graft. The nature of this regulation is under investigation: nitric oxide synthase, indoleamine 2,3-dioxygenase (IDO), IFN-γ-regulated chemokines, and many other potential mechanisms must be considered. The microcirculation is a target of rejection in some models (34,35) but the immunologic mechanism of injury is not clear. We have ongoing studies in hosts with disruptions of various effector mechanisms to resolve this. For example, renal transplants lacking IFN-γ receptors undergo accelerated rejection with typical necrosis in hosts lacking B cells and immunoglobulin(36), indicating microvascular injury by a cell mediated mechanism. This is probably T cell mediated, but a role for NK cells, or for T cells with NK receptors, is an intriguing possibility. NK receptors engaging allogeneic MHC can inhibit effector mechanisms(37), suggesting that the reduced MHC induction could facilitate graft destruction. The observation that cells bearing NK receptors promote rejection in mice lacking CD28 signaling supports the need for donor study of NK receptors(38).

Although the IFN-γ inducible molecules that mediated the protective effect of IFN-γ on liver allografts are not known, one candidate is donor MHC molecules in the graft. MHC induction accompanies rejection and precedes spontaneous acceptance, and lack of IFN-γ-induced MHC expression accompanies necrosis. High levels of MHC expression can potentially neutralize and divert immune effector mechanisms from destroying the graft during the early post-transplant period, perhaps by release of soluble donor MHC class I antigens. Liver allografts produce soluble donor MHC class I antigens(39-41) which have potential for immune modulating effects(42,43). Perhaps the lack of IFN-γ-induced MHC expression on the parenchymal cells leaves the microvascular endothelium to receive the full force of effector mechanisms. The massive parenchymal MHC induction might act as a sink to divert or buffer the effector mechanisms. This situation may have similarities with donor blood transfusion, in which MHC class I and II induction is increased but rejection is reduced(44).

The fact that IFN-γ mediates protection against necrosis by a direct action on the graft does not contradict the potential importance of the direct effects of IFN-γ on T cell homeostasis. IFN-γ promotes activation-induced death and regulates immunodominance in CD8 effector T cells(45,46). CD8+ effector cells are regulated differently in GKO hosts, developing independently of CD4+ cells and CD40-CD40 ligand interactions(47). Thus the phenotype of liver allografts in GKO and GRKO mice may reflect multiple IFN-γ mediated mechanisms. For example, liver allografts in GKO hosts displayed necrosis and increased infiltration, whereas GRKO allografts showed necrosis without increased infiltration. Given that the hosts for GRKO grafts, unlike GKO hosts, have normal IFN-γ production, this observation at face value suggests that the infiltration is controlled by host IFN-γ production acting directly on host lymphocytes in keeping with its known homeostatic functions. (The lower expression of granzyme A, granzyme B, and FasL at some time points in rejecting livers in GKO hosts versus WT hosts is also compatible with altered T cell homeostasis.) However, given the differences in genetic backgrounds between the GKO and the GRKO experiments, this conclusion must remain tentative pending GRKO and GKO transplants across the same histocompatibility differences.

Spontaneous acceptance of liver allografts may be dependent on the fact that, after antigen-specific activation, CTL are intrinsically programmed to undergo contraction independent of antigen clearance(48). This contraction may prevent the destruction of organs when a massive viral infection cannot be cleared(49). The liver transplant is not unique: kidney transplants in mice frequently undergo survival after a rejection episode (50). Spontaneous acceptance of organ transplants is not the absence of rejection, but the ability of the organ to endure infiltration and immune effector mechanisms to permit survival of the tissue and repair of injury as rejection involutes. The greater tendency of liver to undergo spontaneous acceptance probably reflects the addition of the intrinsic advantages of liver (mass, regeneration after injury, dual blood supply, special liver antigen presenting cells) to general properties of T cell effector systems.

The phenotype of grafts rejecting in GKO hosts may turn out to be mediated by multiple discreet IFN-γ- regulated mechanisms rather than by a single mechanism. An impressive number of distinct activities can now be assigned to IFN-γ during rejection, each acting at a specified time and place but often with opposite net effects. Among the early effects are protection from necrosis; MHC induction; induction of chemokine expression (MIG, IP-10, I-Tac); antagonism with IL-4 in "TH1-TH2" type regulation; induction of rejection of class II mismatched skin grafts; induction of enzyme indoleamine 2,3-dioxygenase(51); and homeostasis of effector cell populations. Later in the course, IFN-γ promotes arterial deterioration in some models in which early rejection is suppressed(52,53). (Spontaneously accepted liver transplants do not develop such lesions in our study, at least at 100 days, presumably because of the completeness of the tolerant state.) In this sense, the separate study of IFN-γ receptors in the graft and the host may prove very productive in dissecting the complex pathogenesis of organ graft rejection.

Table 3-1. Sequence of Real-Time PCR primers

Genes	Primer Sequence	
Granzyme A	Forward	5'-ATCTGTGCTGGCGCTTTGA-3'
	Reverse	5'-ACTTAGATCTCTTTCCCACGTTACAGT-3'
	Probe	5'-TGAAAAGAACTGGGTGTTGACTGCTGCC-3'
Granzyme B	Forward	5'-CGATCAAGGATCAGCAGCCT-3'
	Reverse	5'-CTTGCTGGGTCTTCTCCTGTTCT-3'
	Probe	5'-TGCTGCTCACTGTGAAGGAAGTATAATAAATGTCACT-3'
Perforin	Forward	5'-GAAGACCTATCAGGACCAGTACAACTT-3'
	Reverse	5'-CAAGGTGGAGTGGAGGTTTTTG-3'
	Probe	5'-ACCAGGCGAAAACTGTACATGCGACACT-3'
Fas-L	Forward	5'-GAAGGAACTGGCAGAACTCCG-3'
	Reverse	5'-CCCTGTTAAATGGGCCACACT-3'
	Probe	5'-AAAGCAAATAGCCAACCCCAGCACACC-3'
HPRT	Forward	5'-TGACACTGGTAAAACAATGCAAACT-3'
	Reverse	5'-AACAAAGTCTGGCCTGTATCCAA-3'
	Probe	5'-TTCACCAGCAAGCTTGCAACCTTAACC-3'

Table 3-2. Pathology of Liver transplants in WT (BALB/c H-2^d) and GKO (H-2^d) hosts

	CBA into WT			CBA into GKO			p value
Pathology	**Day 5**	**Day 7**	**Day10**	**Day 5**	**Day 7**	**Day10**	
Portal Triad Inflammation	1.2 ± 0.2	2.2 ± 0.4	2.0 ± 0.3	2.4 ± 0.2	3.0 ± 0.0	3.0 ± 0.0	0.02
Venous Inflammation	1.4 ± 0.2	2.2 ± 0.4	1.4 ± 0.2	2.8 ± 0.2	3.0 ± 0.0	2.8 ± 0.4	0.01
Bile Duct Inflammation	1.0 ± 0.0	2.0 ± 1.6	1.6 ± 0.2	2.2 ± 0.2	2.0 ± 0.3	2.4 ± 0.2	0.06
Necrosis	0	0	0	3.2 ± 0.4	3.2 ± 0.2	3.6 ± 0.2	0.005

p value calculated between WT and GKO at day 10 using Mann-Whitney Test.

5 mice in each group

Table 3-3. Pathology of 129 (H-2^b) and GRKO (H-2^b) liver grafts in WT hosts at day 10.

Pathology	129 grafts (n= 5)	GRKO grafts (n= 7)	p value*
Portal Triad Inflammation	1.8 ± 0.4	2.1 ± 0.9	0.43
Venous Inflammation	2.0 ± 0	2.6 ± 0.8	0.07
Bile Duct Inflammation	1.8 ± 0.4	2.6 ± 0.5	0.03
Necrosis	0.4 ± 0.5	3.0 ± 1.2	0.01

*Significant difference between 129 and GRKO was calculated using Mann-Whitney Test

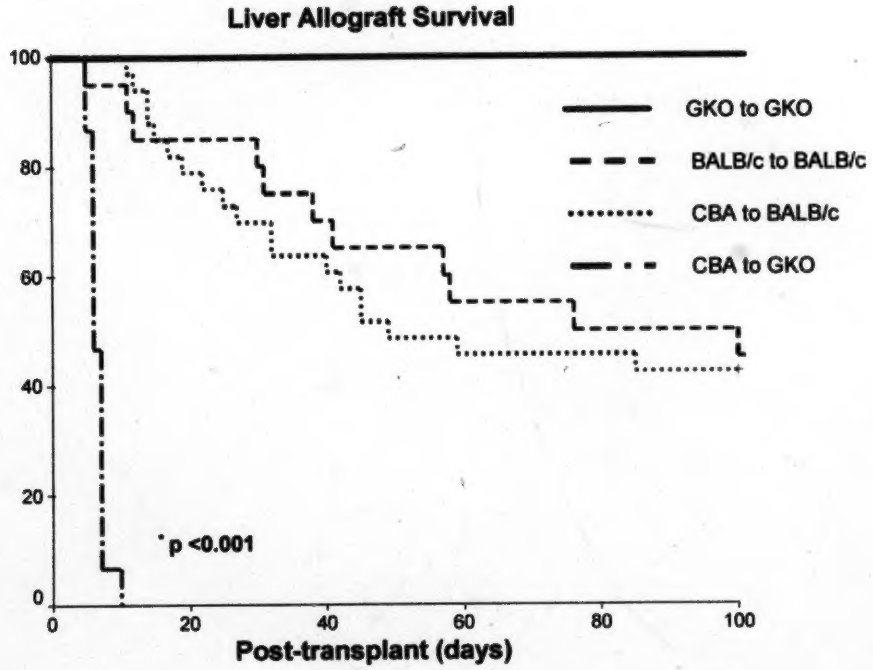


Figure 3-1. Survival of CBA/J (H-2^k) liver allografts in WT (BALB/c, H-2^d) and GKO (H-2^d) hosts, compared to syngeneic grafts. Syngeneic (BALB/c → BALB/c) grafts survived >100 days in 45.0% of mice (n=20). The majority of graft loss in syngeneic transplants was due to common bile duct complications. Survival of liver allograft wild-type hosts (CBA → BALB/c) was 42.2% (n=33) at 100 days post-transplantation which was not statistically different from the survival rate observed in the BALB/c syngeneic transplants (p=0.71). There was no spontaneous acceptance of liver allografts in GKO hosts (CBA → BALB/c IFN-γ -/-) (n=15); no graft survived beyond 14 days post-transplantation. All control syngeneic GKO → GKO (n=4) liver transplants survived beyond 100 days post-transplant.

Figure 3-2.

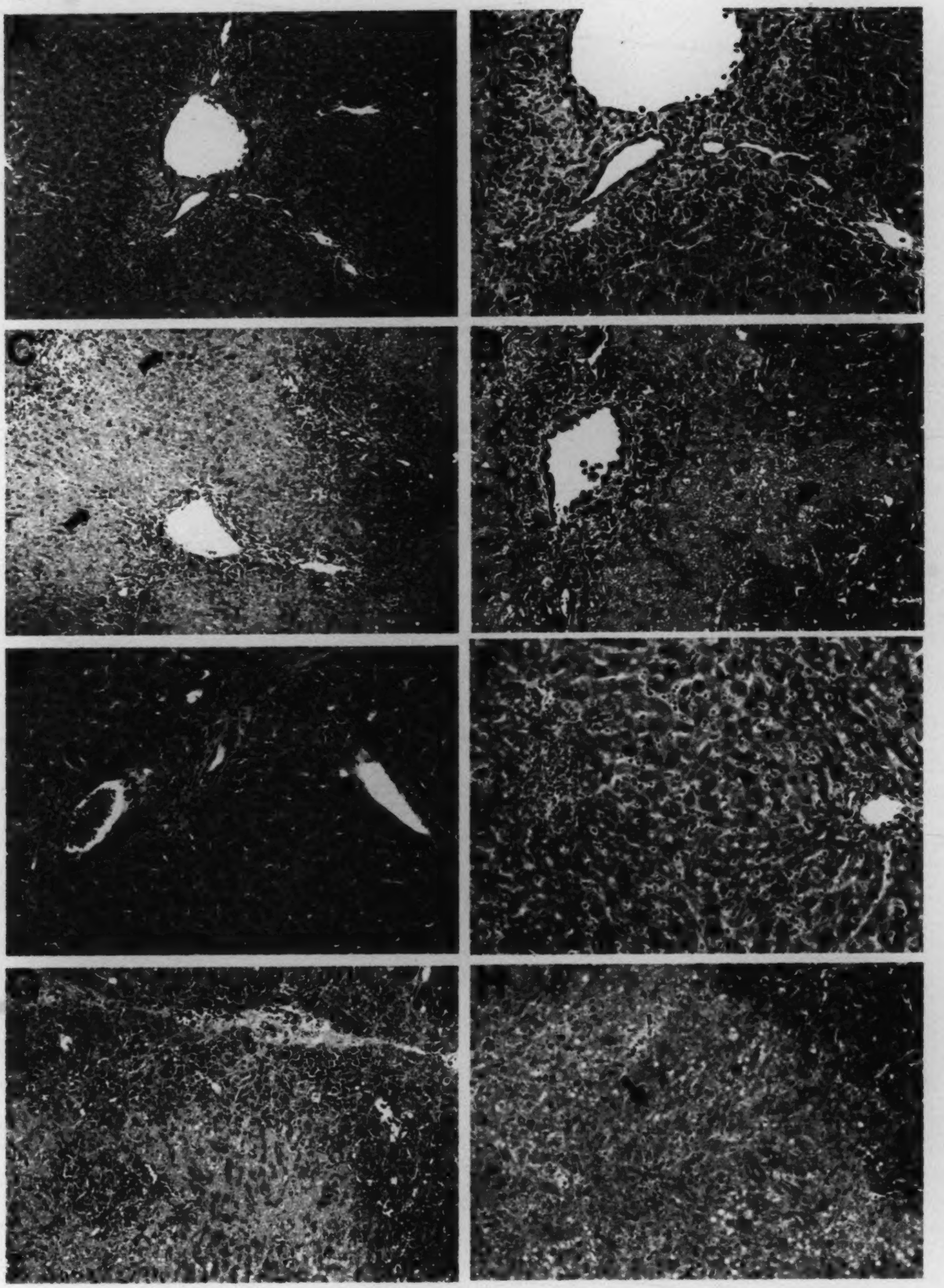


Figure 3-2. Pathology of liver allografts in wild-type and IFN-γ-deficient mice. A, B: CBA/J (H-2^k) liver allografts in WT mice (BALB/c, H-2^d) at day 10 post transplant (A: H&E 100x, B: H&E 200x). C, D: Liver allografts into IFN-γ-deficient mice (GKO, H-2^d), at day 10 post transplant (C: H&E 100x, D: H&E 200x). The arrows indicate areas of necrosis with loss of hepatocytes. E: Liver allografts in WT mice (BALB/c) at > 100 days post-transplant showing mild cellular infiltrate with normal hepatic architecture (E: H&E 200x). F: WT (129/SvJ, H-2^b) liver allografts in CBA hosts at day 10, showing acute rejection (F: H&E 200x). G, H: GRKO (129/SvJ, H-2^b) liver allografts into CBA hosts at day 10 post-transplant, showing infiltrate (G: H&E 200x) and severe necrosis of areas of parenchyma (arrow). (H: H&E 200x).

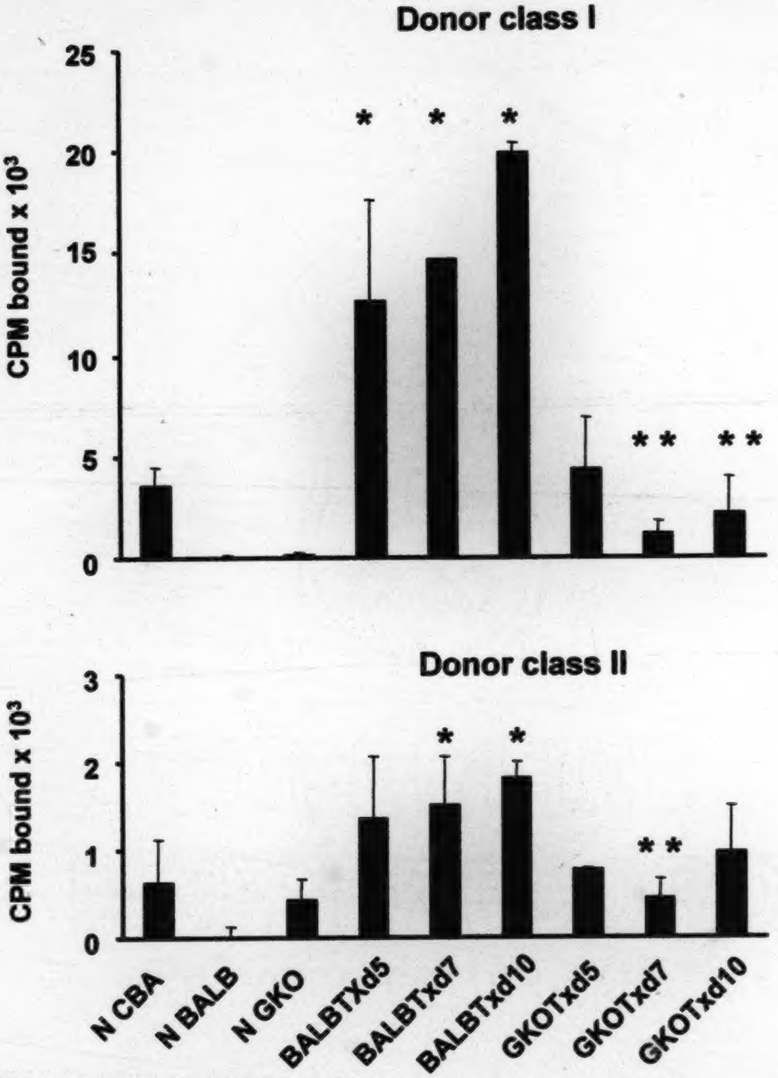


Figure 3-3. MHC Class I and II expression in liver allografts by RABA. Radiolabeled-antibody binding assay measurements of donor MHC class I and class II in liver allografts transplanted into WT (BALB/c H-2^d), and GKO (H-2^d) hosts. Donor MHC class I and II expression remains at basal levels in grafts that are rejected in GKO hosts. * Significant difference compared to normal CBA, ** significant difference between WT and GKO. (*N.B. This assay was performed by Joan Urmson)*

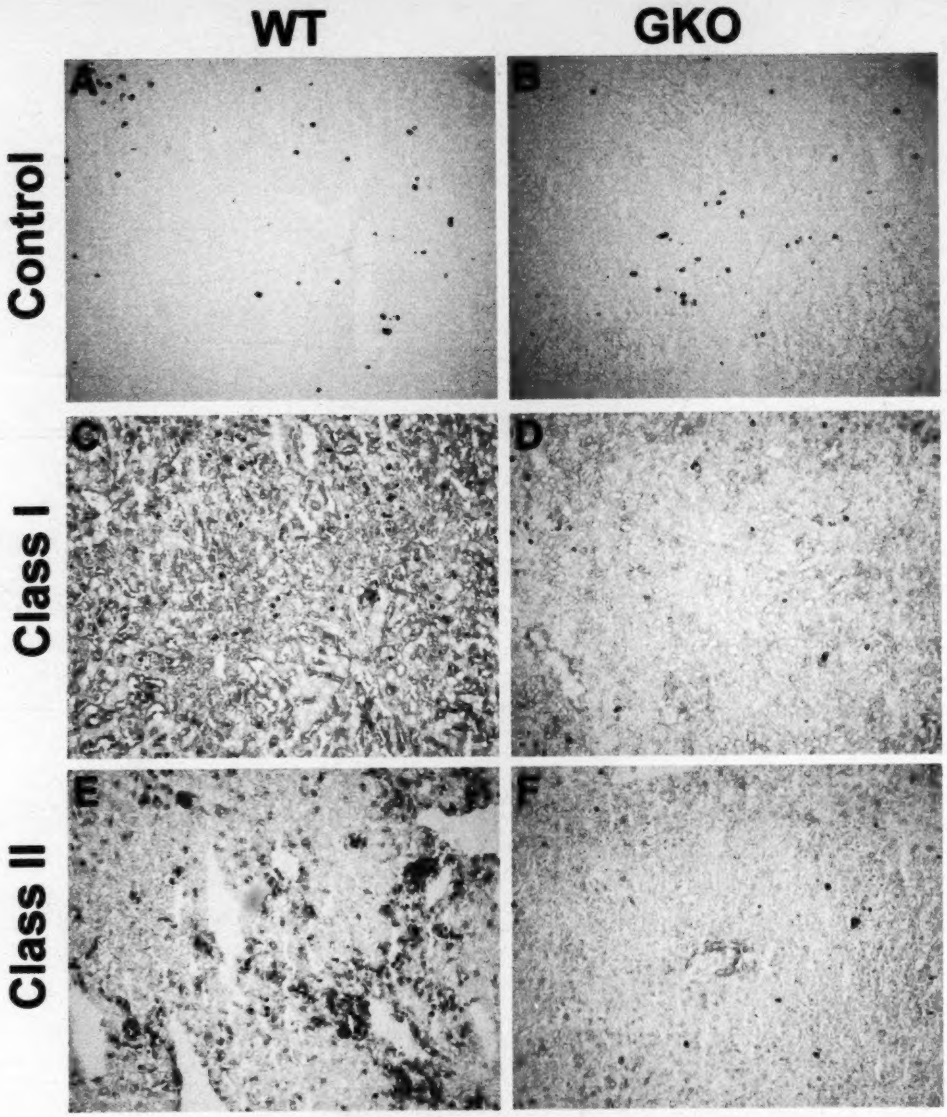


Figure 3-4. MHC Class I and II expression in rejecting liver allografts at day 10 post transplant. (A,B) Control staining of WT (BALB/c, H-2^d) and GKO (H-2^d) hosts. Note peroxidase positive cells which were in the infiltrate of the rejecting livers but were absent from normal livers. (C,D) MHC donor class I (M1) staining of the parenchyma of the graft into WT and GKO hosts. (E,F) MHC class II (M5) staining of grafts in WT and GKO hosts (Magnification 200x). Stained with peroxidase labeled goat anti rat as second antibody. *(N.B. This assay was performed by Joan Urmson)*

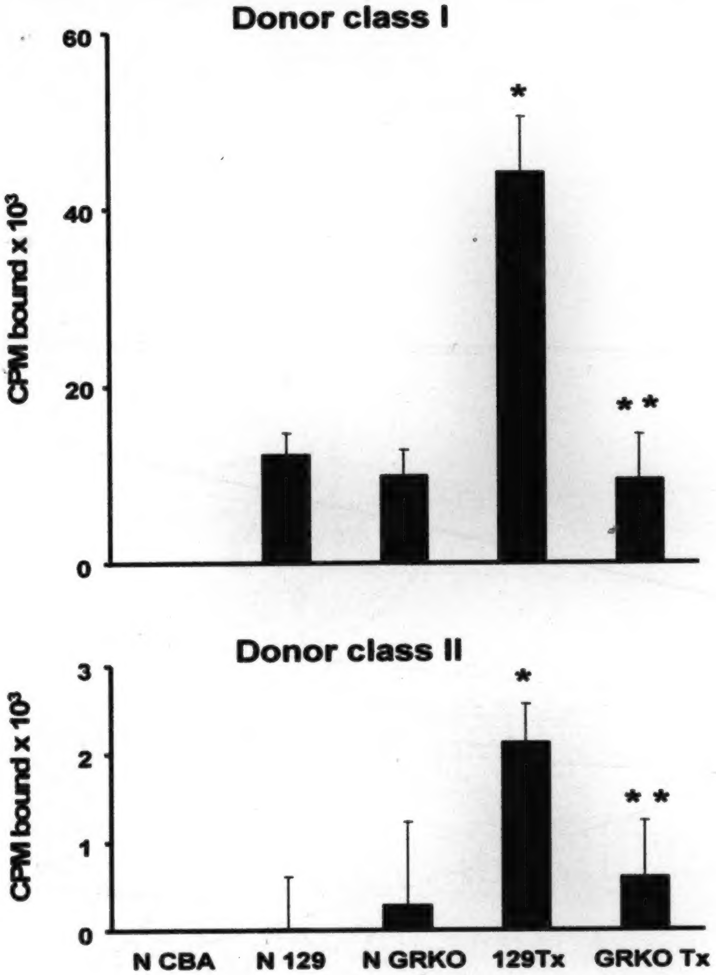


Figure 3-5. Radiolabeled-antibody binding assay of MHC class I and II expression in WT and GRKO grafts. (A) Measurement of donor MHC class I expression demonstrated strong induction of donor MHC class I antigens in wild-type 129/SvJ (H-2^b) grafts transplanted into CBA/J (H-2^k) hosts at post-transplant day 10. GRKO (H-2^b) grafts had no induction of donor MHC class I expression. (B) Donor MHC class II antigens were also increased at day 10 post-transplant in WT grafts, but not in GRKO grafts. * Significant difference compared to normal CBA , ** significant difference between WT and GKRO. (*N.B. This assay was performed by Joan Urmson)*

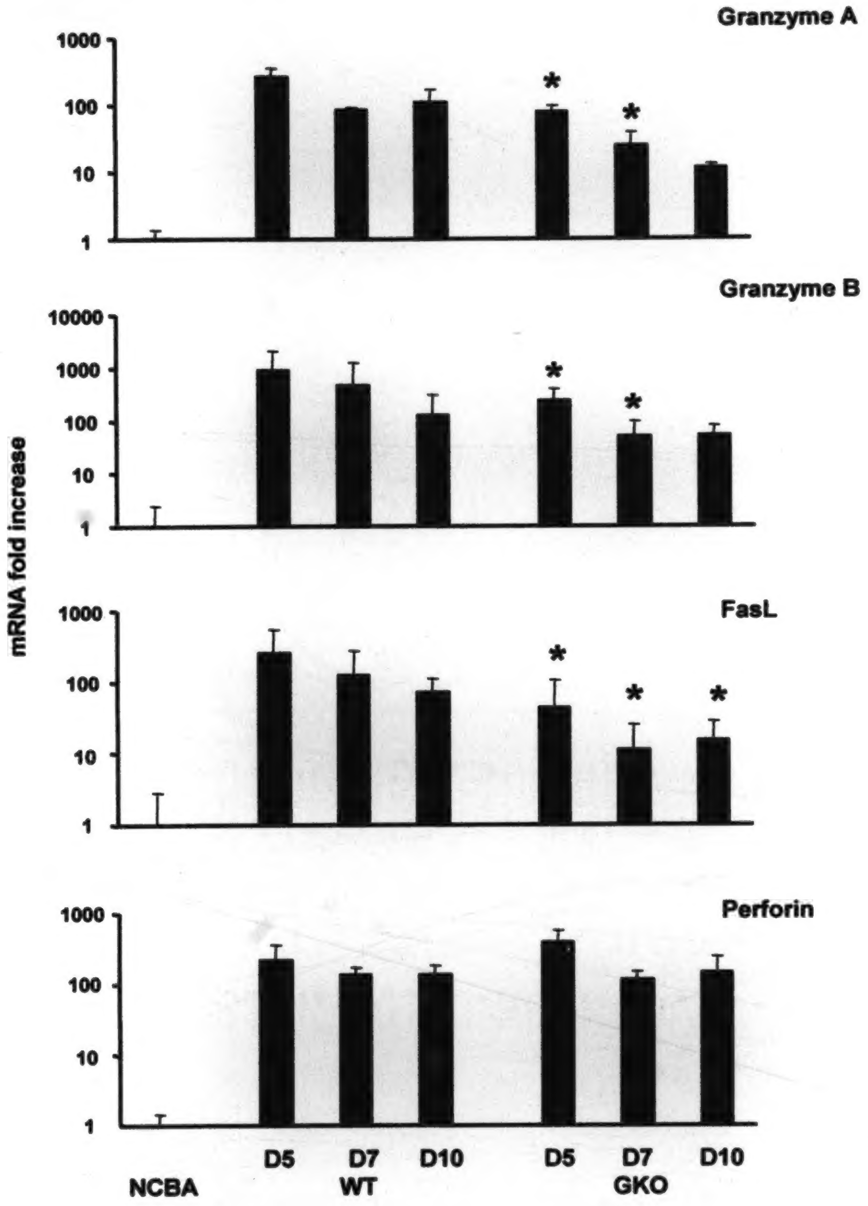
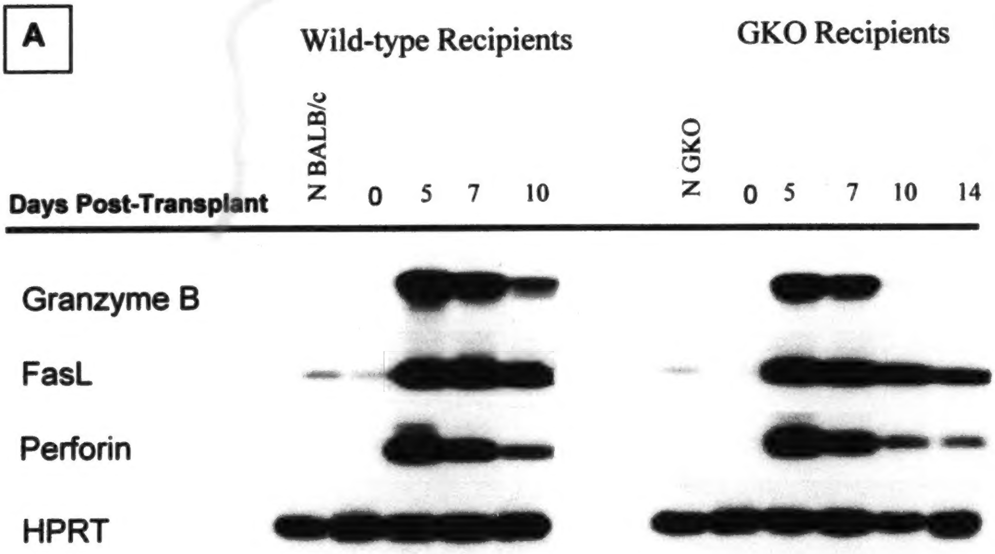


Figure 3-6. Real-time RT-PCR of granzyme A, granzyme B, perforin and FasL expression in liver allografts from WT and GKO mice (H-2^d) at days 5, 7 and 10 post transplant. The mRNA levels are expressed as fold increase over normal donor CBA (H-2^k). * Significant difference between WT and GKO. *(N.B. This assay was performed by B Halloran)*

Appendix of additional assays performed by Tina Mele


A
Wild-type Recipients
GKO Recipients
N BALB/c
N GKO
Days Post-Transplant
0
5
7
10
0
5
7
10
14
Granzyme B
FasL
Perforin
HPRT

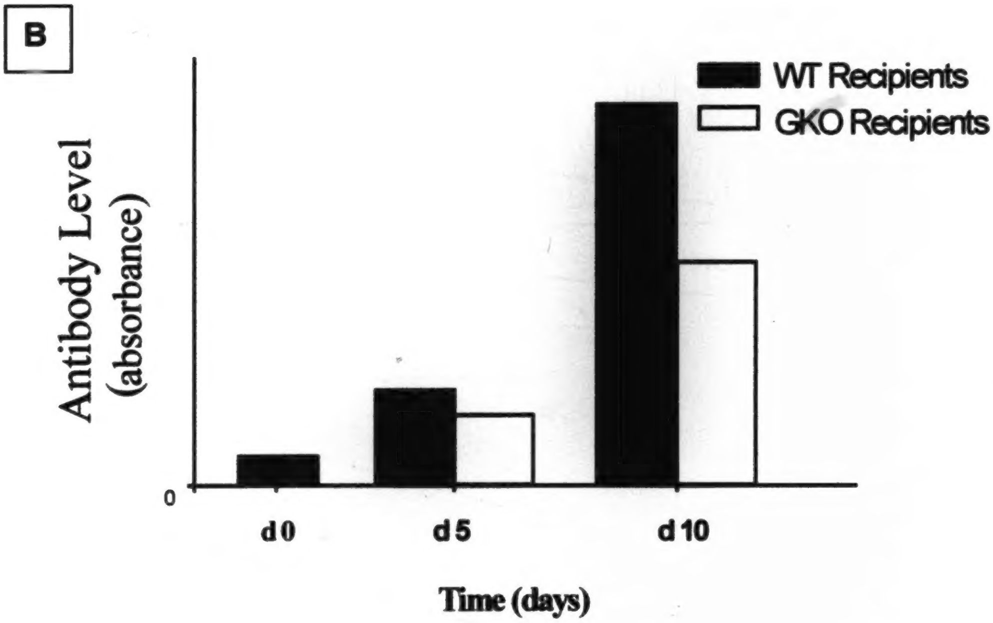


Figure 3-7. Evaluation of systemic immune responses in liver allograft recipients. A. Perforin, granzyme B, and FasL mRNA expression in normal, rejecting and spontaneously accepted livers from WT and GKO recipients. CBA/J livers were transplanted in WT or GKO mice and harvested at days 5, 7 and 10. RT PCR was used to amplify mRNA using sequence specific primers. PCR products were Southern blotted, probed with internal oligomers. HPRT was used as a loading control. Southern blot analysis demonstrated that expression of all three cytotoxic genes was induced post-transplant at similar levels in liver allografts harvested from both WT and GKO mice. Data represents one of three independent experiments. B. Serum anti-donor MHC class I antibody levels in liver allograft recipients. Alloantibodies in sera from WT and GKO liver recipients was measured using an ELISA assay. Both WT and GKO mice produced abundant antidonor antibodies post-transplant. However, in comparison to GKO mice, donor-specific antibodies were increased in sera taken from WT mice who were accepting their allografts. Data represent mean absorbance intensity ± s.e.m (n=4).

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INDUCTION OF MHC CLASS I EXPRESSION PROTECTS LIVER ALLOGRAFTS FROM REJECTION

INTRODUCTION

Spontaneous acceptance is defined as the long-term survival of an allograft in the absence of any immunosuppressive treatment. Liver allografts in experimental animal models are often accepted without the use of immunosuppressive therapy whereas heart, skin and kidney grafts are consistently rejected.[1,2,3] In addition, spontaneously accepted liver allografts have the ability to induce donor specific tolerance such that other organs including hearts, and kidneys can also be transplanted and accepted without the need for immunosuppression.[2,4] Despite intensive research in this area, mechanisms mediating spontaneous acceptance and donor-specific tolerance remain to be identified.

One potential mechanism mediating spontaneous acceptance involves IFN-γ which has been shown to have a protective effect in rejecting renal allografts during the first two weeks post-transplant, preventing graft capillary congestion and ischemic necrosis by a direct action on graft IFN-γ receptors.[5] Our previous experiments demonstrated that livers transplanted into IFN-γ-deficient (GKO) mice are never spontaneously accepted.[6] We also demonstrated that liver allografts lacking receptors for IFN-γ were also uniformly rejected, indicating that the protective effect of IFN-γ occurred by a direct local action on the graft itself.[6] Thus, we concluded that IFN-γ was essential for the spontaneous acceptance of liver allografts.

We sought to identify the molecules that mediate the IFN-γ protective effect. One candidate is donor MHC class I. Our previous work demonstrated that IFN-γ induced the expression of MHC class I antigens in spontaneously accepted grafts whereas in the absence of IFN-γ, there was no induction of IFN-γ and no spontaneous acceptance.[6] Therefore, we hypothesized that the protective effect of IFN-γ occurs by inducing MHC class I expression in the graft. The transporter associated with antigen processing 1 (TAP1) gene encodes a subunit for a transporter involved in the delivery of peptides across the endoplasmic reticulum membrane to MHC class I molecules.[7] Mice with a disrupted TAP1 gene are defective in the stable assembly and intracellular transport of MHC class I and consequently show severely reduced levels of cell surface class I molecules. In the present studies, we determine if the protective effect of IFN-γ is dependent on induction of MHC class I expression in the graft by transplanting MHC class I-deficient (TAP KO) and control C57B/6 liver grafts into normal BALB/c mice. Our results demonstrate that similar to grafts transplanted into IFN-γ deficient mice, there is no spontaneous acceptance of MHC class I-deficient liver grafts supporting the hypothesis that the protective effect of IFN-γ acts via a MHC class I-dependent effector mechanism that occurs locally within the graft.

A possible explanation for the protective effect of IFN-γ may be secondary to apoptosis of graft-infiltrating T cells. Apoptosis ("programmed cell-death") is normally activated in response to physiological signals such as death receptor ligation (FasL or TNF)[8] or withdrawal of survival signals. Activation-induced cell death, one of two pathways leading to apoptosis, is specifically mediated by Fas-FasL following activation of a cell.[9] We hypothesized that spontaneous acceptance occurs as a result of IFN-γ acting directly on the graft to induce MHC class I expression which leads to activation-induced cell death of graft-infiltrating cells. To evaluate our hypothesis, we transplanted liver allografts into WT and IFN-γ-deficient hosts and used TUNEL staining and FACS analysis to evaluate apoptosis of the graft-infiltrating cells. Our results demonstrate an increased number of graft-infiltrating cells undergoing apoptosis in grafts in WT hosts compared to grafts in IFN-γ-deficient hosts supporting our hypothesis that spontaneous acceptance occurs as a result of IFN-γ acting directly on the graft to induce apoptosis of graft-infiltrating cells. In addition, RT-PCR analysis confirmed that spontaneous acceptance of liver allografts was associated with persistent expression of FasL expression.

MATERIALS AND METHODS

Cell lines and reagents

The human T cell lymphoma line Jurkat (American Type Culture Collection (ATCC), Manassa, VA) was grown in RPMI 1640 medium with 2 mM L-glutamine (Gibco BRL Life Technologies, Burlington, ON) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 25 mM HEPES, 100 μM 2-mercaptoethanol, 100 μg/ml penicillin and 100 μg/ml of streptomycin. Jurkat cells stably expressing Bcl-2 gene were a generous gift from Michele Barry (Heritage Medical Research Institute, University of Alberta, Edmonton, Canada).[10] The Jurkat cells transfected with bcl-2 were maintained in RPMI 1640 with 2 mM L-glutamine (Life Technologies, Burlington, ON) supplemented with 10% FBS (Hyclone, Logan, UT), 25 mM HEPES, 100 μM 2-mercaptoethanol, 100 μg/ml of penicillin and 100 μg/ml of streptomycin.

Mice. BALB/c (H-2^d), CBA/J (H-2k), C57BL/6 (B6, H-2^b) and B6.129S2-^{Abcb2tm1Arp} (TAPKO) mice aged 6-12 weeks were obtained from Jackson Laboratories, Bar Harbor, ME. The Abcb2 deficient mice were created on a C57BL/6 background and were obtained as homozygotes.[7] IFN-γ-deficient (GKO) mice were generated as described previously[11]. Briefly, a normal allele in mouse embryonic cells was replaced with a defective gene using a targeted vector which introduced a termination codon after the 30 first amino acids of the mature IFN-γ protein. These stem cells were used to construct mice heterozygous for the disrupted gene, which were intercrossed and the progeny were selected for homozygosity. Breeding pairs of IFN-γ deficient mice with disrupted IFN-γ genes (GKO mice, H-2^d) were a generous gift from Dr. Tim Stewart (Genentech Inc., South San Francisco, CA) and a colony is maintained at HSLAS, University of Alberta, Edmonton, Canada. The Health Sciences Laboratory Animal Services at the University of Alberta maintained all mice. All experimental procedures were in agreement with animal care protocols enforced by the institution review board.

Orthotopic liver transplants. Livers from male mouse donors (C57BL/B6, CBA and TAPKO) were isolated and transplanted into 9-11 week old male BALB/c mice according to a newly developed model of mouse orthotopic liver transplantation with hepatic arterialization. Briefly, under isoflurane anaesthesia, a transverse abdominal incision was followed by dissection and division of the portal and supra-hepatic veins, hepatic artery, and common bile duct and removal of the liver. The host mice were similarly anaesthetized and the donor liver implanted orthotopically with re-anastomosis of the portal and supra-hepatic veins and hepatic artery. The common bile duct was reconstructed over a polyethylene stent. Mice receiving liver grafts were monitored daily for evidence of graft failure (jaundice, decreased activity, and weight loss). Graft loss as a result of rejection was confirmed by histologic examination of tissues obtained at autopsy. No immunosuppressive therapy was given to any mice during the course of the study. Spontaneous acceptance was defined as liver allograft survival at 100 days following transplantation in the absence of immunosuppressive drug therapy. Statistical analysis of spontaneous acceptance rates in each of the strain combinations was performed using Kaplan-Meier statistical analysis (SPSS, Chicago, Ill.).

Pathology. Severity of liver allograft rejection was determined using the Banff grading system12 which assigns cellular infiltration of portal triads, bile duct inflammation and endothelial inflammation a score of 1-3; 1, minimal changes; 2, moderate changes; or 3, severe changes. The extent of necrosis was scored as the percentage of parenchyma involvement (0, no necrosis present; 2, 25-50% of total parenchyma involved; 3, 50-75% of the total parenchyma involved and 4, >75% of total parenchyma involved). Mann-Whitney statistical analysis was used to compare the severity of histologic lesions seen in WT and TAP KO grafts transplanted into WT mice.

TUNEL assay. *In situ* terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) analysis was done on paraffin-embedded 3 μm sections of liver tissue to detect apoptotic cells at various time points following transplantation based on a technique of Gavrieli et al[13]. Briefly, liver sections were deparaffinized in xylene and hydrated through a series of alcohols. To inactivate endogenous peroxidase, the sections were immersed in 1% H_2O_2 for 8 minutes, and then rinsed with distilled water. The sections were treated with proteinase K (20 µg/ml in PBS) for 10 minutes and rinsed with PBS. The sections were air dried, then flooded with TdT (terminal deoxynucleotide transferase) (Hoffmann-LaRoche, Quebec) buffer at room temperature for 30 minutes. The slides were then incubated at 37^0C in a humidified chamber for 1 hour with TdT buffer (30 mM Tris HCl (pH 7.2), 1 mM $CaCl_2$, 140 mM sodium cacodylate) containing 0.25 nmol/µl biotin-16-dUTP and 0.25 U/µl TdT to label the nicked ends of DNA. The slides were then washed twice with PBS. To block nonspecific staining, the slides were incubated with 2% bovine serum albumin in PBS for 20 minutes at room temperature and followed by two washes in PBS. We then incubated the slides with an avidin-peroxidase complex (Vector Laboratories) for 30 minutes and then washed twice in PBS. 3,3'-diaminobenzidine tetrahydrochloride substrate was used to visualize the reaction and the slides were counterstained with methyl blue and mounted with Permount (Fischer, Nepean, ON). *In situ* TUNEL staining of mouse thymus was used as a positive control for the assay. For negative controls, TdT was omitted. Apoptotic cells were counted in 10 high-power fields (original magnification x 400) in each liver graft. Data is expressed as a mean ± SE.

Assessment of FasL expression: Total RNA was extracted from individual liver samples according to a modification of the method described by Chomczynski et al.[14] Briefly, liver graft tissue obtained at various times post-transplant, frozen in liquid nitrogen and stored at –70°C, was homogenized in 4M guanidinium isothiocyanate, and the RNA was pelleted through a 5.7M $CsCl_2$ cushion. RNA concentrations were determined by UV absorbance at 260 nm. RNA was transcribed into cDNA using Superscript reverse transcriptase (BRL, Burlington, Ontario) and amplified in a Perkin Elmer Cetus thermal cycler using Taq DNA polymerase using sequence specific primers for the FasL gene (Sense 5'-GATTCCTCAAAATTGATCAGAGAGAG-3', Antisense: 5'- GATTCCTCA AAATTGATCAGAGAGAG-3', Probe: 5'-CAAATAGCCAACCCCAGTACACCCTC TGA-3'). The PCR products were Southern blotted using 10 μL of DNA electrophoresed through a 1.5% agarose gel with transfer to nylon nitrocellulose filters. DNA was cross-linked to the nylon filter using ultraviolet irradiation. Blots were then hybridized with ^{32}P-labelled cDNA probes for FasL. The Southern blots were then phosphoimaged. HPRT was used as a loading control. Data is expressed as a mean ± SE.

Isolation of graft-infiltrating cells

To characterize cells infiltrating the grafts, we isolated the cells from grafts in WT hosts at day 7 post-transplantation according to a modification of the method described by Sharland et al[15]. Briefly, the liver graft recipient was anesthetized using inhalation halothane anesthesia. A transverse abdominal incision is made. The portal vein was identified and cannulated with a 22-gauge catheter. The catheter was secured in situ using an 8-0 silk ligature suture. The liver was perfused with 10 cc of Hanks' Balanced Salt Solution (HBSS) (Gibco BRL Life Technologies, Burlington, ON) to remove blood within the vasculature. Two ml (1 mg/ml of collagenase IV, Sigma Chemical Co., St Louis, MO) of collagenase solution is then perfused via the catheter. The mouse was then euthanized and the liver graft excised and placed in a Petri dish. The liver was perfused by the catheter with HBSS to ensure complete removal of circulating blood. The graft was gently diced into small pieces using scissors and placed into a 50 mL Falcon tube with 20 mL of collagenase solution. The tube was placed in a shaker bath at 37 degrees for 30 minutes to allow for digestion of the graft. The cell suspension was gently teased and ground on a sterile nylon mesh (200μm). The cell suspension was washed twice in RPMI 1640 medium with L-glutamine (Gibco BRL Life Technologies, Burlington, ON) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 25 mM HEPES, 100 μg of penicillin/ml and 100 μg of streptomycin/ml at (400g) 1800 rpm for 5 minutes. The pellet containing hepatocytes, lymphocytes and other living cells was resuspended in 7 ml of Percoll solution (density 1.079) (Sigma Chemical Co., St Louis, MO) and centrifuged at 39, 000g for 10 minutes at 4^0C. The top layer consisting of intact and fragmented hepatocytes was removed. The middle layer, containing the nonparenchymal cells, was pipetted into a separate tube and washed once with RPMI 1640 at 400g for 5 minutes. The cells were counted using the Trypan blue exclusion method.

Isolation of infiltrating cells from spleens

To characterize cells present within the spleens of liver graft recipients, we isolated the cells from the spleens at day 7 post-transplantation at the time of liver allograft isolation. Briefly, the liver graft recipient was anesthetized using inhalation halothane anesthesia. A transverse abdominal incision is made. The liver graft was removed as described above. The spleen was then isolated and immediately the spleen tissue was ground through a fine wire screen in RPMI 1640. The cells were then pelleted. Red blood cells were lysed with buffered ammonium chloride lysis buffer. The cells were then washed twice with RPMI. The cells were counted using the Trypan blue exclusion method.

Flow Cytometric Analysis

Once the cells infiltrating the liver graft and spleen were isolated, we then proceeded to characterize the cells using FACS analysis. Briefly, we added 50 µL of FITC-labeled anti-H2Kk (donor) or anti-H2Dd (recipient) rat IgG2a monoclonal antibody (Cedarlane, diluted with PBS to optimized concentration, Current Protocols in Immunology) to 3 ml plastic tubes. We then added 50 µl of liver cell suspension (10^6 cells) to each plastic tube. We incubated the mixture for 30 minutes on ice in the dark. We then added 1ml of cold PBS, centrifuged the cell suspension at 300 x g for 5 minutes at 4^0 C. We aspirated the supernatant from the cell pellet and washed the cells twice with cold PBS. These steps were repeated for Cytochrome-labeled anti-CD3, CD4, CD8, CD19 (rat IgG2a) or biotinylated anti-pan NK (IgM) monoclonal antibodies (Cedarlane, diluted with PBS to optimized concentration). The cells were resuspended in 4% paraformaldehyde in PBS and incubated for 30 minutes at room temperature. The cells were pelleted and washed in cold PBS. We then prepermeablized the cells in 100 µl of staining buffer (PBS + 1%FBS + 0.1% NaN_3 + 0.1% saponin, Perm/Wash, Pharmingen) and incubated for 5 minutes. We assessed for apoptosis by detecting active caspase-3 activity within the cells. We washed the cells with PBS and then resuspended in staining buffer with optimized dilution of PE-conjugated anti-active caspase-3 antibody (polyclonal rabbit IgGs, Cedarlane) and then incubated for 30 min at room temperature in the dark. The cells were then washed once in PBS, resuspended in 250 µl of cold PBS and analyzed via three-color flow cytometry by examining 10 000 events on a FACScan flow cytometer using CELLQuest software (Becton Dickinson, Mississauga, Ontario, Canada). Cells deemed FITC-H-2k or H-2d, PE-active caspase 3 or cytochrome-CD3, CD4, CD 8, CD19, CD 45 positive were those displaying fluorescence greater than fluorescence of the isotype antibody controls (data shown in appendix). Jurkat cells pretreated with 6 µM camptothecin (Sigma Chemical Co., St Louis, MO), a known inducer of apoptosis,[16] for 4 hours and Jurkat cells transfected with Bcl-2 were as positive and negative controls for detection of apoptosis, respectively.

RESULTS

Spontaneous acceptance of liver allografts requires TAP expression

We transplanted syngeneic (BALB/c, H-2^d) and allogeneic (C57Bl/6, H-2^b and TAP KO, H-2^b) livers into BALB/c hosts. Long-term survival of BALB/c livers transplanted into BALB/c mice (n=21) was 52.4% at 100 days post-transplant. The majority of graft loss in these syngeneic transplants was the result of common bile duct complications. During the early post-transplant period, two mice died of bile duct leaks (day 5 and day 11) and a third mouse died at day 12 from multiple liver abscesses. Later in the post-transplant period, most grafts were lost due to biliary obstruction secondary to ischemia resulting in biliary duct stenosis. When allogeneic WT (C57Bl/6) liver grafts were transplanted into BALB/c mice (n=19), the spontaneous acceptance rate was 42.1% at 100 days post-transplant (p=0.29). Thus few allogeneic WT liver grafts were lost to rejection. In contrast, there is no spontaneous acceptance of MHC class I-deficient liver grafts (n=9) compared to WT liver allografts (p< 0.001) (Figure. 4-1). These results are similar to what was previously demonstrated with WT liver grafts transplanted into IFN-γ-deficient hosts with no spontaneous acceptance of liver allografts in the absence of IFN-γ.[6] Therefore, induction of MHC class I expression is required for spontaneous acceptance of liver allografts and may account for the protective effect of IFN-γ observed in WT liver transplants.

Histology

Histologic analysis of control, wild-type C57BL/6 grafts following transplantation demonstrates the hallmarks of rejection; mononuclear cells infiltrating the bile ducts and endothelialitis of the arteries and veins (Figure 4-2A, B and C). Despite severe rejection activity and increasing parenchymal necrosis (Table 4-1), WT grafts survive through this initial rejection period and progress onto long-term survival and spontaneous acceptance. TAP KO liver allografts at day 10 post-transplantation demonstrate a mononuclear cellular infiltrate expanding the portal triad with no parenchymal necrosis (Figure 4-2 D, E). At day 15 post-transplant, a control C57BL/6 liver graft demonstrates severe rejection with an increased cellular infiltrate and increased parenchymal necrosis compared to day 10 post-transplant (Figure 4-2C). In contrast, a TAP KO liver at day 18 post-transplant demonstrates moderate rejection similar to the TAP KO liver at day 10 post-transplant with no evidence of parenchymal necrosis (Figure 4-2F). Thus, during the initial period post-transplant the pathology and rejection severity of liver allografts is similar in both the wild-type C57BL/6 and MHC class I-deficient grafts despite the difference in long-term outcome.

Assessment of Apoptosis of Infiltrating Cells in Liver Allografts

CBA/j liver allografts transplanted into WT and GKO mice were harvested at days 5, 7 and 10 post-transplant to measure apoptosis by TUNEL assay. *In situ* TUNEL staining demonstrates undetectable levels of apoptosis in a normal resting liver (Figure 4-3B) whereas staining of a CBA/j graft taken from a WT host at day 7 demonstrates many apoptotic cells in the cellular infiltrate around the portal triads where rejection is focused in liver transplantation (Figure 4-3C, D) with few apoptotic cells in the parenchyma. In contrast, *in situ* TUNEL staining of a graft taken from a GKO host at day 7 demonstrates fewer apoptotic graft-infiltrating cells (Figure 4-3E, F). The number of apoptotic cells in each graft was counted in 5 high power fields and expressed as a mean. TUNEL analysis of liver grafts harvested from both WT and GKO recipients demonstrated significantly higher levels of apoptotic graft-infiltrating leukocytes in grafts taken from WT recipients compared to grafts from GKO recipients at day 7 post-transplant (11.7±1.7 vs 4.8 ±0.4; $p < 0.05$, Table 4-2). At day 10 following transplantation, there is no statistical difference in the number of apoptotic cells detected in grafts harvested from WT and GKO recipients. This observation may be explained by several reasons. Apoptosis is a short-lived process that generally requires approximately 3 hours from initiation to completion. Analysis of a liver allograft by TUNEL staining is really just a "snapshot" in time of a dynamic process that is rapidly recurring within the graft and analysis of cells infiltrating the graft qualitative at best. In addition, it is well-documented that spontaneously accepted liver allografts undergo a period of early rejection followed by resolution and acceptance. The liver allograft may be undergoing resolution of the rejection period with accommodation of the graft by day 10 post-transplant. In addition, the difficulty in obtaining adequate number of surviving GKO grafts beyond day 7 following transplantation prevented a proper statistical analysis at these later time points. As expected, the TUNEL+ cells in liver allografts were predominantly located in periportal areas, within the region of concentrated leukocyte infiltrate, suggesting that apoptosis occurs predominantly in graft-infiltrating leukocytes. Thus, spontaneous acceptance of WT liver allografts is associated with increased number of apoptotic graft-infiltrating cells compared to IFN-γ-deficient liver allografts. This suggests that the protective effect of IFN-γ may occur by inducing apoptosis of graft-infiltrating cells and allowing recovery from the initial rejection period.

Characterization of infiltrating cells within liver allografts and spleens isolated from WT recipients

To further analyze the cells possibly mediating spontaneous acceptance, we harvested liver grafts and spleens from WT hosts at day 7 post-transplant and isolated the infiltrating cells. The cells were then incubated with fluorescent antibodies and analyzed via three-color flow cytometry. The cells were identified based on cell origin (donor vs recipient), cell type (CD3, CD4, CD8, CD19 or CD45) and apoptosis activity (anti-caspase 3 activity). Not surprisingly, the majority of cells infiltrating the graft were of recipient origin (Table 4-3). CD8 and CD4 cells compromised 43.64% and 28.6% of the cell population, respectively (Table 4-3). More importantly, at day 7 post-transplant there was a trend towards increased frequency of apoptosis of recipient cells compared to donor cells (4.21% vs 1.45%) which supports the TUNEL staining results. Thus, spontaneous acceptance of liver allografts in WT hosts is associated with apoptosis of infiltrating recipient cells.

Assessment of FasL expression in liver allografts

To determine if spontaneous acceptance was associated with persistent FasL expression since antigen-induced cell death occurs via FasL or TNF, we harvested grafts at various time points following transplantation and performed RT-PCR analysis of mRNA isolated from the liver grafts. RT-PCR analysis of FasL mRNA demonstrated persistently elevated levels of FasL expression in spontaneously accepted liver allografts (Figure 4-4). Although there was initial elevated expression of FasL in grafts from WT hosts, given there was no survival beyond 14 days post-transplant, we were unable to determine FasL expression beyond day 14 in GKO hosts. These results are consistent with those described previously by Pan et al in spontaneously accepted rat allografts.[17] They demonstrated that localization of FasL expression gradually switched from graft-infiltrating cells to hepatocytes as the graft gradually overcome rejection and became accepted. Conversely, Fas was expressed strongly on infiltrating lymphocytes and weakly on hepatocytes at day 14 post-transplantation. Thus, persistent expression of FasL in spontaneously accepted liver allografts is consistent with ongoing antigen-induced cell death occurring within these grafts.

DISCUSSION

Although most liver allografts undergo a period of rejection during the first two weeks post-transplantation, there is a high rate of spontaneous acceptance of allografts in most strain combinations. Typically, histologic examination reveals a mononuclear cellular infiltrate of the portal triads that peaks at two weeks and gradually resolves over time. Hepatocytes demonstrate numerous mitotic figures indicating regeneration of these cells. By 4 weeks, sinusoidal mononuclear cells have disappeared and those cells remaining in the portal triads are lymphocytes. By 3-4 months, the histology appears normal. Thus, spontaneous acceptance is not associated with a failure to initiate an immunologic response, but is the result of termination of rejection at an early stage.

Our results demonstrate that in the absence of induction of donor MHC class I, there is no spontaneous acceptance of liver allografts. MHC class I molecules are expressed on all nucleated cells, and the major function of these molecules is to present peptide fragments of antigens to T cells. The known ligands of the MHC class I-peptide complex are the T cell receptor, the coreceptor CD8, the lectin-like receptors, the killer cell immunoglobulin (Ig)-like receptors (KIRs), and other molecules codified in the leukocyte receptor complex of Ig-related genes[18,19,20,21,22]. However, there are many reports suggesting that aggregation of MHC class I is able to induce positive and negative intracellular signals in T and B lymphocytes as well as in NK cells, resulting in tyrosine phosphorylation of multiple proteins,[23,24,25] increases of intracellular Ca^{2+}, interleukin (IL)-2 production and proliferation,[26,27] T cell apoptosis,[28,29] inhibition of T and B cell activation[30,31] and inhibition of NK cell lytic activity.[32] These data suggest that MHC I could not only be ligands of antigen-recognition and signaling receptors but also signaling molecules by themselves, likely through membrane colocalization with supramolecular activation clusters[33] or association with coreceptors more directly involved in signal transduction.

One possible explanation for the protective effect of donor MHC class I expression involves high membrane MHC class I expression which delivers a negative signal to infiltrating effector lymphocytes, preserving the integrity of the graft. Natural killer (NK) cells would be likely candidates for negative regulation by high MHC expression. NK cells are cytotoxic effector lymphocytes able to recognize and to induce the lysis of a variety of target cells, including primarily virus-infected cells as well as tumor cells.[34,35] Multiple families of receptors regulate NK cell cytotoxicity. The interactions of these receptors with their ligands control different inhibiting/activating signal pathways, and it is the balance of these signals that determines the behavior of the NK cell. It is well recognized that NK cells recognize major histocompatibility complex (MHC) class I molecules through surface receptors [lectin-like receptors and killer cell immunoglobulin (Ig)-like receptors (KIRs)], delivering signals that inhibit NK cell function.[36,37] Hence, NK cells lyse those target cells that have lost or express insufficient amounts of MHC I proteins. In the absence of IFN-γ, there is lack of MHC class I induction and thus, grafts would be vulnerable to unregulated attack by NK cells.

Some investigators have postulated that acceptance of liver grafts is due to deletion of alloreactive cells. However, despite in vivo hyporesponsiveness to liver allografts, splenocytes or liver lymphocytes isolated from mice with an accepted liver allograft display donor-specific reactivity in vitro during mixed leukocyte reactions and cytotoxicity assays. This phenomenon, described as "split tolerance", argues against clonal deletion of alloreactive T cells as the mechanism involved in spontaneous acceptance.[38,39] An ongoing, active process such as antigen-induction cell death (AICD) would be a more likely mechanism for spontaneous acceptance.

Apoptosis, or "programmed cell-death", is normally activated in response to physiological signals such as death receptor ligation (FasL or TNF) or withdrawal of survival signals. Antigen-induced cell death, one of two pathways leading to apoptosis, is specifically activated by chronic antigen stimulation of a cell. A possible mechanism for spontaneous acceptance may occur as a result of IFN-γ acting directly on the graft to induce MHC class I expression which leads to repeated chronic antigen stimulation of graft-infiltrating cells which causes FasL on donor leukocytes in the graft to engage Fas on the graft-infiltrating cells which then leads to multiple downstream events leading to the characteristic DNA fragmentation observed in apoptotic cells. This hypothesis is supported by evidence in previously published studies. Induction of allograft tolerance has been demonstrated to be impaired in various mouse strains that have profound defects in activation-induced cell death. For example, IL-2 knockout mice, defective in activation-induced T cell death,[40,41,42,43,44] are resistant to induction of tolerance to islet and cardiac allografts by rapamycin treatment[45] or co-stimulation blockade.[46] Rapamycin, in contrast to calcineurin inhibitor CsA, blocks growth factor-imparted proliferative signals[47] but does not block antigen priming for activation-induced cell death[48]. Furthermore, blockade of both signal 1 and signal 2 of T cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance[49] supporting the theory that apoptosis of alloreactive T cells is an essential initial step for induction of allograft tolerance. Costimulation B7-CD28 (signal 2) blockade leads to long-term allograft acceptance.[50,51] CTLA4Ig, a recombinant fusion protein, blocks the B7-CD28 T cell costimulatory pathway (signal 2) and induces long-term allograft survival in wild-type rodents.[52,53,54,55] CTLA4 engagement has been demonstrated to be crucial for the peripheral tolerance induced by blocking the CD28 T cell costimulatory pathway[56]. However, CTLA4Ig treatment of IL2 -/- mice on day 2 post-transplantation did not produce long-term cardiac allograft acceptance.[46] In addition, this study demonstrated that CTLA4Ig increases alloantigen-driven T lymphocyte apoptosis. Although TCR ligation in the absence of costimulation has been shown to induce T cell anergy in vitro,[57] deletion of alloantigen-specific T cells may constitute another mechanism by which the B7-CD28 blockade induces long-term allograft survival.[58, 59] Furthermore, Konieczny et al demonstrated that IFN-γ is critical for the long-term allograft survival that is induced by blocking the CD28 and CD40 ligand costimulation pathways.[60] These studies support the hypothesis that activation-induced apoptosis of alloreactive T cells occurs during long-term allograft survival.

The increased number of apoptotic cells in grafts transplanted into WT hosts compared to GKO hosts is interesting given that there are relatively fewer cells infiltrating the graft in WT hosts compared to grafts in GKO recipients, suggesting that this may be due to apoptosis of the alloreactive T cells in the WT grafts. The persistent FasL expression in spontaneously accepted liver grafts is also consistent with ongoing antigen-induced cell death. Our results demonstrate that the protective effect of IFN-γ during the spontaneous acceptance of liver allografts is a result of IFN-γ acting directly on the graft and requires induction of donor MHC class I expression. Our results demonstrate an increased number of graft-infiltrating cells undergoing apoptosis in grafts in WT hosts compared to grafts in IFN-γ-deficient hosts supporting our hypothesis that spontaneous acceptance occurs as a result of IFN-γ acting directly on the graft to induce apoptosis of graft-infiltrating cells. IFN-γ-induction of donor MHC class I may lead to activation-induced apoptosis of alloreactive T cells and promote spontaneous acceptance of liver allografts.

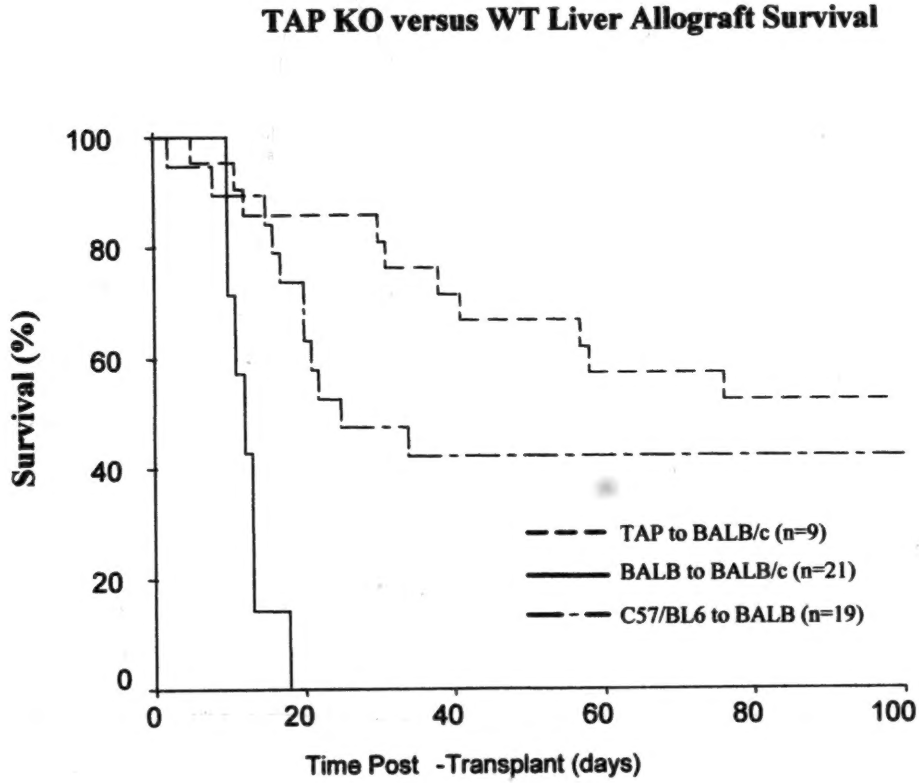

Figure 4-1: Survival of WT (C57Bl/6, H-2b) and TAP KO (H-2b) liver allografts in BALB/c (H-2d) hosts compared to syngeneic grafts.

Syngeneic (BALB/c into BALB/c) grafts survived >100 days in 52.4% of mice (n=21). The majority of graft loss in syngeneic transplants was the result of common bile duct complications. Survival of WT liver allografts into BALB/c hosts was 42.1% (n=19) at 100 days post-transplantation which was statistically not significant from the survival observed in the BALB/c syngeneic transplants (p=0.29). There was no spontaneous acceptance of TAP KO grafts (n=9); no graft survived beyond 18 days post-transplant.

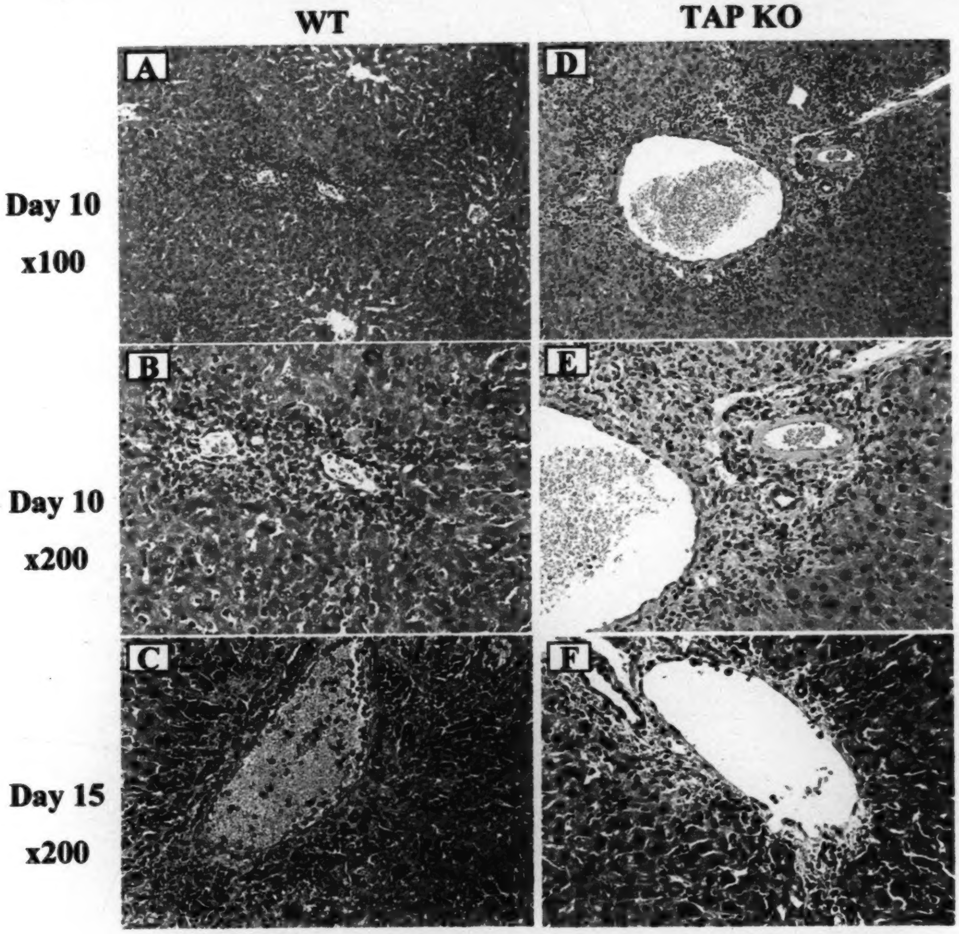

Figure 4-2. Histopathology of rejecting WT and TAP KO mouse allografts in WT hosts. (A, B) Wild-type C57Bl/6 (H-2b) liver allograft at day 10 post-transplantation demonstrating a mononuclear cellular infiltrate expanding a minority of the portal triads with minimal parenchymal necrosis (A x100, B x200). (D, E) TAP KO (H-2b) liver graft at day 10 post-transplantation demonstrating an increased mononuclear cellular infiltrate expanding the portal triad with no evidence of parenchymal necrosis (D x100, E x200). (C) Wild-type C57BL/6 liver graft at day 15 post-transplant demonstrating severe rejection with an increased cellular infiltrate and increased parenchymal necrosis compared to day 10 post-transplant (x200). (F) TAP KO liver at day 18 post-transplant demonstrating moderate rejection similar to the TAP KO liver at day 10 post-transplant with no evidence of parenchymal necrosis (x200).

Table 4-1. Pathology of WT (C57Bl/6, H-2^{b}) and TAP KO (H-2^{b}) liver transplants in WT (BALB/c H-2^{d}) hosts.

Pathology	**day 5 (n=4)**	**day 10 (n=4)**	**day 15 (n=4)**
Portal Triad Inflammation			
WT	2	3	3
TAP KO	2	2	3*
Venous Inflammation			
WT	1	2	1
TAP KO	2	1	1*
Bile Duct Inflammation			
WT	2	2	1
TAP KO	2	1	1*
Rejection Activity Index			
WT	5	7	5
TAP KO	6	4	5*
p value	NS	NS	NS
Necrosis			
WT	1	2	3
TAP KO	1	2	1*

p value calculated using Mann-Whitney test. NS: not significant

* n=1 for TAP KO survival at day 15

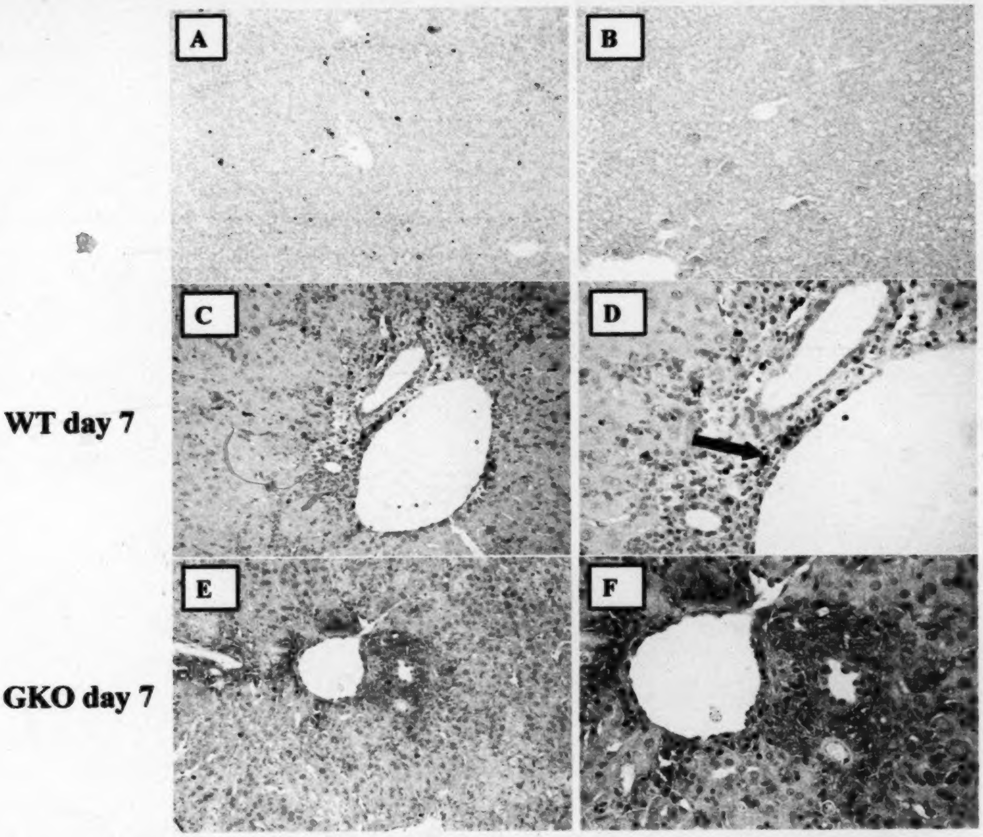

Figure 4-3. TUNEL staining of liver allografts from WT and GKO hosts at day 7 post-transplant.

In situ TUNEL staining of wild-type CBA/j thymus tissue demonstrates basal state levels of apoptosis and served as a positive control since apoptosis occurs during T cell selection (Figure 4-3A, original magnification x100). *In situ* TUNEL staining of wild-type CBA/j liver tissue demonstrates undetectable levels of apoptosis in a normal resting liver (Figure 4-3B, original magnification x100) whereas staining of a CBA/j graft taken from a WT host at day 7 demonstrates many apoptotic cells (black arrow) in the cellular infiltrate around the portal triads where rejection is focused in liver transplantation (Figure 4-3C, D; C original magnification x200, D original magnification x400). In contrast, *in situ* TUNEL staining of a graft taken from a GKO host at day 7 demonstrates fewer apoptotic graft-infiltrating cells (Figure 4-3E, F; E original magnification x200, F x original magnification x400).

Table 4-2. TUNEL staining of apoptotic cells in liver allografts from WT versus GKO recipients

	Day 5 cells/hpf (± SEM) (n=3)	Day 7 cells/hpf (± SEM) (n=3)	Day 10 cells/hpf (± SEM) (n=3)
WT	16.1 ± 2.5	11.7 ± 1.7	6.6 ± 0.2
GKO	18.2 ± 0.7	4.8 ± 0.4	4.2 ± 1.9
p value	NS	$p < 0.05^{*}$	NS

All data are shown as mean ± SE.

Mann-Whitney test used to determine significance.

NS: not statistically significant.

Table 4-3. Phenotypic Analysis of Infiltrating Cells Isolated from Liver Allograft and Recipient Spleen in WT host at day 7 post-transplantation.

	Liver		**Spleen**	
	Donor Cells (%)	**Recipient Cells (%)**	**Donor Cells (%)**	**Recipient Cells (%)**
Total Isolation	8.04	69.79	3.2	78.23
CD3	9.97	57.94	0.46	32.93
CD4	5.01	28.60	0.30	14.35
CD8	7.67	43.64	0.35	17.21
CD19	1.51	3.55	0.08	30.93
CD45	9.61	73.32	2.1	79.91
Active Caspase	1.45	4.21	0.46	5.43

NB. Representative results of one of 3 experiments

Table 4-4. Phenotypic Analysis of Infiltrating Cells Undergoing Apoptosis Isolated from Liver Graft in WT host at day 7 post-transplantation.

	Donor Cells (%)	**Recipient Cells (%)**
CD3	21.55	59.50
CD4	13.81	22.33
CD8	15.15	71.89
CD19	6.94	4.93
CD45	26.83	93.23

NB. Representative results of one of 3 experiments

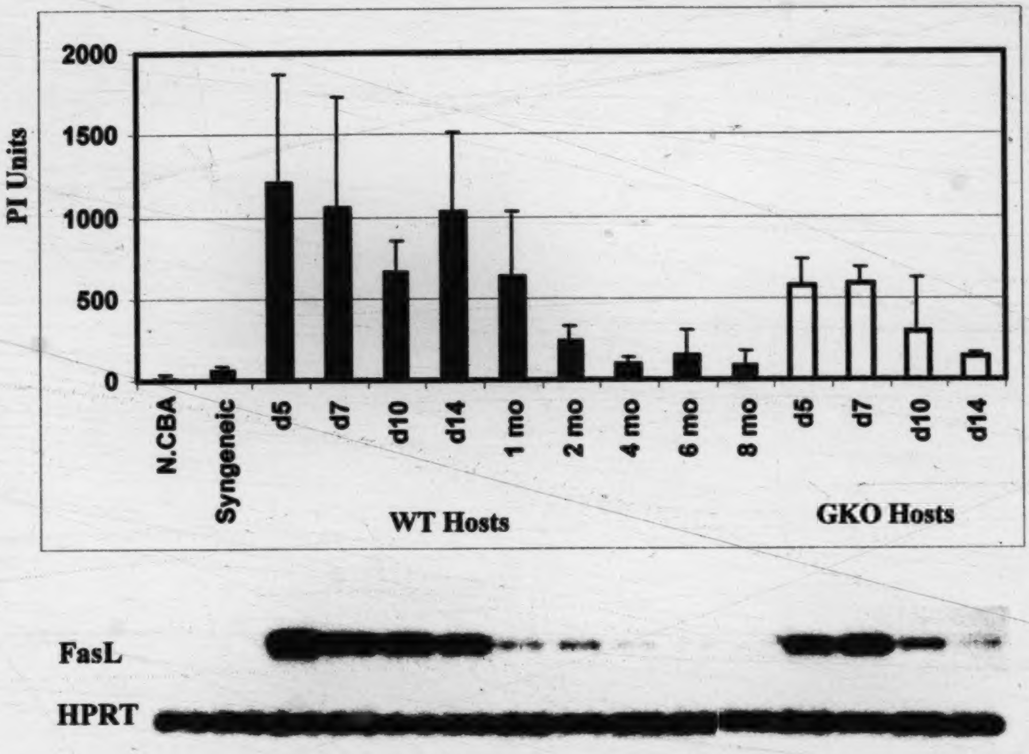

Figure 4-4. The evaluation of mRNA for FasL in spontaneously accepted and rejecting grafts from WT and GKO hosts.

RT-PCR analysis of FasL mRNA demonstrated persistently elevated levels of FasL expression in spontaneously accepted liver allografts in WT hosts. PCR products were Southern blotted and probed with internal oligomers. The blots were then phosphoimaged. The data is presented as mean ± SE. HPRT was used as a loading control.

Appendix

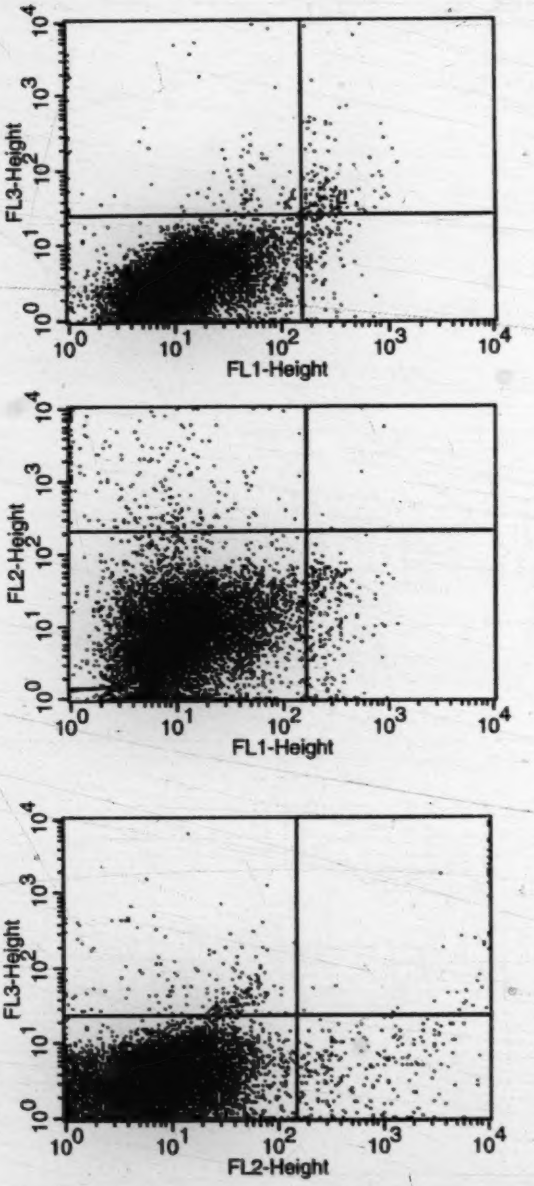

Figure 4-5. Isotype controls for three color FACs analysis. Cells were incubated with all three isotype control antibodies for FITC-labeled, Cytochrome-labeled and PE-labeled antibodies. FL1: FITC channel, FL2: PE channel, FL3: Cytochrome channel

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SUMMARY CHAPTER

The success of organ transplantation has been brought about with advancements in several areas of medicine. Namely, understanding of the immunology involved in transplantation and rejection, the development of various surgical techniques and the discovery of immunosuppressive drugs have greatly increased the success rate of human organ transplantation. Liver transplantation remains the only definitive treatment for end-stage liver disease, however, the complications due to liver allograft rejection and generalized immunosuppression persist lifelong. The ultimate goal in solid organ transplantation is to achieve a state of immunologic tolerance between donor and recipient, eliminating entirely the need for drugs. This dream of tolerance has been driven by evidence both from experimental animal models[1] and in humans[2] that the liver itself can produce an immunologic tolerant state that may reduce or potentially eliminate the need for immunosuppressive substances. Despite intensive research since Owen's first observations of immunologic tolerance in freemartin cattle, the mechanisms involved in spontaneous acceptance of allografts remain to be identified.

Experimental liver allografts undergo spontaneous acceptance despite undergoing rejection during the first few weeks post-transplant. Thus, spontaneous acceptance is not associated with failure to initiate an immunologic response, but is the result of the ability of the organ to endure cellular infiltration and immune effector mechanisms to permit survival of the tissue and repair of injury as rejection involutes. The greater tendency of the liver to undergo spontaneous acceptance almost certainly reflects, in part, the intrinsic advantages of the liver including its larger tissue mass relative to other organs, its ability to regenerate after injury, its dual blood supply and unique resident antigen presenting

Kupffer cells. The effects of IFN-γ in the spontaneous acceptance of liver allografts was unknown prior to our research and of considerable interest given the unique features of the liver.

The studies of liver allograft rejection in hosts lacking IFN-γ and in livers from donors lacking IFN-γ receptors establish that a direct effect of IFN-γ on the graft is an absolute requirement for the spontaneous acceptance of liver allografts. There were no survivors if IFN-γ was not present. In comparison, if IFN-γ was present the survival of MHC-incompatible livers was similar to that of the syngeneic controls, in other words, complete spontaneous acceptance. Comparison of liver rejection in WT and GKO hosts established that IFN-γ affected several aspects of the early rejection phenotype: it reduced hemorrhage, necrosis and cellular infiltration while inducing high MHC expression. The phenotype of rejecting liver allografts lacking IFN-γ receptors was similar to that observed with grafts transplanted into GKO hosts. Specifically, there was severe rejection with extensive necrosis of the parenchyma in GRKO grafts and no spontaneous acceptance. Therefore, grafts that lacked receptors for IFN-γ and thus, could not be regulated by IFN-γ, were never spontaneously accepted. Thus, the protective effect of IFN-γ was secondary to a direct action on the graft itself.

We sought to identify the mechanisms that mediate the protective effect of IFN-γ. One candidate is donor MHC class I. Our initial experiments demonstrated that IFN-γ induced the expression of MHC class I antigens in spontaneously accepted grafts whereas in the absence of IFN-γ, there was no induction of MHC class I and no spontaneous acceptance. Therefore, we hypothesized that the protective effect of IFN-γ occurs by inducing MHC class I expression in the graft. We examined the role of MHC class I induction in spontaneous acceptance by transplanting MHC class I-deficient (TAP KO) into normal BALB/c mice. Similar to grafts transplanted into IFN-γ deficient mice, there is no spontaneous acceptance of MHC class I-deficient liver grafts. Our results demonstrate that donor MHC class I molecules in the graft perform an essential role in the spontaneous acceptance of liver allografts supporting the hypothesis that the protective effect of IFN-γ acts via a MHC class I-dependent effector mechanism that occurs locally within the graft.

One possible explanation for the protective effect observed with MHC class I expression is that high levels of MHC expression potentially neutralize and divert immune effector mechanisms from destroying the graft during the early post-transplant period, perhaps by release of soluble donor MHC class I antigens. Liver allografts produce soluble donor MHC class I antigens[3,4,5] which have potential for immune modulating effects.[6,7] The massive parenchymal MHC induction might act as a sink to divert or buffer the effector mechanisms. This situation may have similarities with donor blood transfusion, in which donor MHC class I and II induction is increased but rejection is reduced.[8]

A second possible explanation for the protective effect seen with MHC induction involves regulation and/or inhibition of natural killer (NK) cells. NK cells are cytotoxic effector lymphocytes able to recognize and to induce the lysis of a variety of target cells, including primarily virus-infected cells as well as tumor cells.[9,10] The interactions of NK receptors with their ligands control different inhibiting/activating signal pathways, and it is the balance of these signals that determines the behavior of the NK cell. It is well recognized that NK cells recognize MHC class I molecules through surface receptors [lectin-like receptors and killer cell immunoglobulin (Ig)-like receptors (KIRs)], delivering signals that inhibit NK cell function.[11,12] High membrane MHC class I expression delivers a negative signal to infiltrating effector NK cells, preserving the integrity of the graft whereas reduced MHC induction observed in GKO hosts could facilitate graft destruction since NK cells lyse those target cells that have lost or express insufficient amounts of MHC I proteins. Therefore, in the absence of IFN-γ there is lack of MHC class I induction and thus, grafts would be vulnerable to unregulated attack by NK cells.

Apoptosis ("programmed cell-death") is normally activated in response to physiological signals such as death receptor ligation (FasL or TNF)[13] or withdrawal of survival signals. Antigen-induced cell death (AICD), one of two pathways leading to apoptosis, is induced by antigen stimulation under particular conditions. Repeated T cell stimulation by antigen results in engagement of death receptors and activation of caspase-8.[14,15,16] In CD4+ T cells the major death receptor responsible for triggering AICD is Fas.[17,18,19] *In vitro* experiments have previously demonstrated that activated CD4+ T cells lacking IFN-γ are resistant to AICD.[20] In this study, IFN-γ induced AICD by stimulating the expression of caspases downstream of the Fas death receptor through the transcriptional activity of Stat1. Similarly, in our model, IFN-γ may exert its protective effect by inducing apoptosis of graft-infiltrating T cells. Spontaneous acceptance may occur as a result of IFN-γ acting directly on the graft to induce MHC class I expression which leads to AICD of graft-infiltrating cells and FasL-mediated apoptosis. This hypothesis is supported by Qian et al who demonstrated increased apoptosis of infiltrating cells within spontaneously accepted liver allografts.[21] When acute liver allograft rejection was induced by intraperitoneal administration of IL-2 from days 0 to 4 post-transplantation, apoptotic activity of nonparenchymal cells was substantially reduced. Furthermore, previously published studies supports the possibility that apoptosis of graft-infiltrating cells mediated through activation of the Fas/FasL system might account for the immune privilege of certain tissues such as testis[22] and the anterior chamber of the eye.[23] Although rodent and human livers do not normally express CD95L,[24,25] CD95L mRNA is found within hepatocytes in inflammatory liver disease.[26] Furthermore, rodent LEW (RT1.^{1}) liver allografts are spontaneously accepted when transplanted into fully MHC-mismatched DA (RT1.$^{\mathrm{aaav1}}$) recipient rats. Following transplantation into DA recipients, LEW hepatocytes are induced to express high levels of FasL with low levels of Fas expression.[27] Induction of hepatocyte FasL expression is dependent on transplanting across a MHC-mismatch since transplantation of LEW liver grafts into syngeneic LEW hosts does not result in hepatocyte FasL expression. Furthermore, depletion of the donor passenger leukocyte population by 10-Gy whole body irradiation prior to transplantation abrogates the tolerance induced by the liver graft[28] and prevents induction of hepatocyte FasL expression.[29] Our results of persistent FasL expression in spontaneously accepted liver grafts are consistent with those described previously by Pan et al in spontaneously accepted rat allografts.[30] They demonstrated that localization of FasL expression gradually switched from graft-infiltrating cells to hepatocytes as the graft gradually overcome rejection and became accepted. Conversely, Fas was expressed strongly on infiltrating lymphocytes and weakly on hepatocytes at day 14 post-transplantation. Therefore, there is a clear relationship between the expression of FasL on hepatocytes, apoptosis of infiltrating cells and spontaneous acceptance. Furthermore, blockade of both signal 1 and signal 2 of T cell activation has also been shown to prevent apoptosis of alloreactive T cells and abrogate induction of peripheral allograft tolerance[31] supporting the theory that apoptosis of alloreactive T cells is an essential initial step for induction of allograft tolerance.

Future Studies

Further studies to verify the essential role of IFN-γ in spontaneous acceptance should include administration of recombinant IFN-γ to GKO hosts to document re-establishment of long-term survival of liver allografts. However, administration of recombinant IFN-γ to GKO hosts may not readily prevent necrosis, as shown in previous studies.[32] This may reflect the fact that production of IFN-γ in the rejecting graft is massive and possibly paracrine, acting on contiguous cells, and may be difficult to simulate by systemic IFN-γ administration. We have also attempted to prevent liver allograft necrosis by injecting rIFN-γ intraperitoneally in GKO recipients but as yet with limited success (unpublished results).

To strengthen the association between apoptosis and spontaneous acceptance, restoration of spontaneous acceptance of grafts in GKO hosts could be tested by treating GKO hosts with soluble FasL protein that cross-links the T-cell receptor and thus, activate apoptosis.

However, the major hurdle with this study would be similar to that observed with recombinant IFN-γ treatment of GKO hosts such that large quantities of the FasL protein would be necessary to activate the large population of alloreactive T cells infiltrating the graft. Alternatively, treatment of WT hosts with soluble FasL decoys or $F(ab')_2$ anti-Fas antibodies which are known to block FasL-mediated apoptosis[33] and should prevent spontaneous acceptance if present in sufficient quantity to neutralize Fas on graft-infiltrating cells.

Furthermore, transplantation of liver allografts using mice with mutations for Fas (lpr/lpr) and FasL (gld/gld) would also provide insight into the role of FasL-mediated apoptosis during the spontaneous acceptance of liver allografts. Transplantation of grafts with mutations for FasL genes into normal hosts would prevent induction of FasL on hepatocytes and passenger leukocytes post-transplantation and should abrogate spontaneous acceptance if indeed FasL-mediated apoptosis is required for spontaneous acceptance of liver allografts. Alternatively, transplantation of normal grafts into Fas-deficient hosts would also prevent spontaneous acceptance as the recipient alloreactive T cells would be deficient in Fas and therefore, be immune to FasL-mediated activated-induced apoptosis.

Our preliminary FACS analysis of the cells infiltrating the spontaneously accepted grafts confirm that the majority of the cells in the graft are of recipient origin and approximately 5% of these cells are undergoing apoptosis at any given time. Additional studies that would provide valuable information and further support our initial FACS results could include in situ studies of liver grafts in WT and GKO hosts using confocal microscopy with fluorescent-labeled antibodies for H-2 markers to determine cell origin (recipient versus donor), cell surface markers (specifically CD4, CD8, CD19 and CD11c) and anti-caspase-3 (apoptosis activity).

In summary, our results demonstrate that IFN-γ and induction of donor MHC are critical for the induction of spontaneous acceptance of liver allografts. In addition, the protective effect of IFN-γ during the spontaneous acceptance of liver allografts is a result of IFN-γ acting directly on the graft and is associated with induction of donor MHC class I expression in the graft. Persistent FasL expression and apoptosis of infiltrating donor cells is increased in spontaneously accepted allografts compared to rejecting allografts in IFN-γ-deficient hosts. Therefore, IFN-γ-induction of donor MHC class I may lead to activation-induced apoptosis of alloreactive T cells and promote spontaneous acceptance of liver allografts.

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